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THE ROLE OF OSTEOCALCIN IN HUMAN BONE METABOLISM AND GLUCOSE HOMEOSTASIS

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ACADEMIC DISSERTATION

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2. List of original publications

This thesis is based on the following publications:

I. **Paldánius PM**, Ivaska KK, Hovi P, Andersson S, Kajantie E, Väänänen HK Mäkitie O. *The Effect of Oral Glucose Tolerance Test on Serum Osteocalcin and Bone Turnover Markers in Young Adults*. Calcif Tissue Int (2012): Volume 90 (2): 90-95

II. **Paldánius PM**, Ivaska KK, Hovi P, Andersson S, Kajantie E, Väänänen HK Mäkitie O. *Total and Carboxylated Osteocalcin Associate with Insulin Levels in Young Adults Born with Normal or Very Low Birth Weight* PLoS One (2013) May 3; 8(5): e63036.

III. Viljakainen H, Ivaska KK, **Paldánius P**, Lipsanen-Nyman M, Saukkonen T, Pietiläinen KH, Andersson S, Laitinen K, Mäkitie O. *Suppressed bone turnover in obesity: a link to energy metabolism?* A case-control study. J Clin Endocrinol Metab (2014) Jun;99(6):2155-63

IV. **Paldanius PM**, Ivaska KK, Mäkitie O, Viljakainen H. *Normal reference ranges for serum and urinary osteocalcin in healthy Finnish children and adolescents* (manuscript submitted, poster presentation on April 27, 2015 at ECTS in Rotterdam, The Netherlands)

The publications are referred to in the text by their roman numerals, which also correspond with the independent study cohorts used in this thesis. The corresponding articles were reprinted with the kind permission of the copyright holders. In addition, some previously unpublished data are presented.

3. List of abbreviations

25-OHD	25-hydroxyvitamin D, calcidiol
AGE	Advanced glycation products
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
βCTX-I	Beta carboxy-terminal collagen crosslink
BAP	Bone-specific Alkaline Phosphatase
BF%	Body Fat % (percentage)
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMI	Body Mass Index
BSP	Bone Sial Protein
BTMs	Bone Turnover Markers
CI	Confidence interval
cOC	Carboxylated osteocalcin
Crea	Creatinine
CV	Cardiovascular
D_3	Calcitriol (Vitamin D), also 1,25-dihydroxycholecalciferol /1,25 $(OH)_2D_3$
DPD	Deoxypyridinoline crosslink
DXA	Dual-energy X-ray absorptiometry
ELISA	Enzyme-linked immunoassay
ELLU	Elintavat ja luusto nuorilla
FGF-23	Fibroblast growth factor -23
FN	Femoral neck

FPG	Fasting plasma glucose
fS-INS	Fasting serum insulin
GA	Gestational age
GH	Growth hormone
GLA	γ-carboxyglutamic acid
GPRC6A	G protein-coupled receptor family C group 6 member A
HAP	Hydroxyapatite
HbA1c	Glycosylated haemoglobin A1c
HDL	High density lipoprotein
HOMA-IR	Homeostasis Model Assessment-Insulin Resistance
HUS	Helsingin ja Uudenmaan Sairaanhoitopiiri (Helsinki and Uusimaa Hospital District)
ICTP	Serum Type I collagen carboxyterminal telopeptide
IFG	Impaired Fasting Glucose
IGF-1	Insulin-like Growth Factor 1
IGT	Impaired Glucose Tolerance
IVGTT	Intravenous glucose tolerance test
LDL	Low density lipoprotein
LS	Lumbar spine
Mab	Monoclonal antibody
NGT	Normal glucose tolerance
NTX	N-terminal cross-linked telopeptide of type I collagen
OC	Osteocalcin
OGTT	Oral Glucose Tolerance Test
OPG	Osteoprotegerin

OR	Odd's Ratio
OST-PTP	Osteotesticular protein tyrosine phosphatase
PO_4	Phosphate
P1NP	N-terminal propeptide of type I collagen
PICP	C-terminal propeptides of type I collagen
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
PYD	Pyridinoline crosslink
RANK(L)	Receptor activator of NF-kappa B (ligand)
RIA	Radio-immuno assay
SE	Standard error (of the mean)
SD	Standard deviation
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglyceride
TRACP-5b	Tartrate- resistant acid phosphatase
TSH	Thyroid Stimulating Hormone
TZD	Thiazolidinedione
unOC	Under/uncarboxylated osteocalcin
U-(mid)OC	Urinary mid-fragment osteocalcin
uNTx	Urinary NTx
VLBW	Very Low Birth Weight
WB BA	Whole Body Bone Area
WHO	World Health Organisation
WT	Wild Type (animals)

4. Abstract

Serum osteocalcin (OC) is an osteoblast-derived protein and an established biomarker of bone turnover and formation. Previous studies have focused on its role as a predictor of fractures and only recently, OC has been recognized as an endocrine factor potentially regulating glucose tolerance and energy metabolism. Uncarboxylated OC has been shown to induce expression of adiponectin, insulin, and markers of pancreatic islet cell proliferation in mice while only few studies have evaluated these interactions in humans.

The association of OC and glucose homeostasis has been retrospectively explored, in multiple *post hoc* analyses and in cohorts impacted by multiple confounding factors including chronic metabolic conditions, cardiovascular (CV) co-morbidities, and diabetes. None of the studies have addressed the alleged association in apparently healthy young adults or those with early signs of insulin resistance but no diabetes. In addition, the timing, duration and magnitude of serum OC response to glucose loads in any population remain unknown.

Adult subjects born with very low birth weight (VLBW) present with significant, simultaneous impairments in skeletal health such as lower bone mineral density (BMD), and progressively worsening glucose tolerance in early adulthood. This provides an opportunity to further explore the recently suggested bidirectional regulative pathway between glucose and bone metabolism. The thesis studies evaluated whether early signs of insulin resistance and impaired glucose tolerance in early adulthood in an apparently healthy VLBW cohort, in obese young adults without diabetes, or their controls, could demonstrate if and how the changes in bone and glucose metabolism are associated and whether these changes are reflected in serum OC concentrations and its degree of carboxylation. Furthermore, normal paediatric reference ranges and their independent determinants for serum total, carboxylated and urinary OC were to be established.

No association between parameters of glucose homeostasis and OC were established at any time-point. Energy metabolism evidently influences the key parameters of bone turnover but the direct role of insulin as the mediator of these changes needs further investigations. Contradictory to the preclinical rodent data, serum OC appears to be associated with long-term glucose regulation, if any, whereas acute changes during OGTT may be mediated via other mechanisms.

Age, height and weight, and parathyroid hormone (PTH) and puberty, are independent determinants of serum and urinary OC levels during childhood and adolescence. Circulating OC reflects more pubertal growth status than instant grade of bone mineralisation and thus its validity, similar to other bone turnover markers, as a determinant of bone status in healthy individuals is limited.

With greater discrimination of the different forms of osteocalcin present in circulation and inclusion of multiple measures of bone turnover and glucose homeostasis, evidence currently does not support OC as a protein critical to the regulation of energy metabolism. OC is still a prodigious marker of bone turnover and for monitoring of the effect treatments targeting impaired bone metabolism, especially in adults.

5. Tiivistelmä

Seerumin osteokalsiini on luusolujen tuottama endokriininen merkkiaine, joka kuvastaa luuston aineenvaihduntaa. Tuoreissa tutkimuksissa on havaittu osteokalsiinilla olevan mahdollisesti yhteyttä myös energia-aineenvaihduntaan. Hiirillä osteokalsiinin karboksyloimaton muoto lisää adiponektiinin ja insuliinin eritystä sekä haiman kasvua kuvaavien merkkiaineiden määrää. Näitä piirteitä ei kuitenkaan ole juurikaan tutkittu ihmisillä. Yksittäisissä kohorttitutkimuksissa on tutkittu osteokalsiinin ja glukoositasapainon yhteyttä. Tutkittavat ovat kuitenkin yleensä kärsineet sairauksista, jotka myös vaikuttavat sokeritasapainoon (mm. metabolinen oireyhtymä, diabetes, sydän- ja verisuonisairaudet), ja siten tulosten tulkinta on ollut haastavaa. Terveillä nuorilla aikuisilla sokeritasapainon ja osteokalsiinin yhteyksiä ei ole tutkittu eikä myöskään ole tutkimustietoa ravinnon tai glukoosi-infuusion vaikutuksesta osteokalsiinin erityksen.

Pikkukeskosina syntyneillä todetaan varhaisella aikuisiällä täysiaikaisena syntyneitä ikätovereitaan matalammat luun mineraalitiheydet ja alentunut insuliiniherkkyys. Väitöskirjassa kartoitettiin luuston ja glukoosiaineenvaihdunnan muutosten yhteyttä, sekä osteokalsiinin ja sen karboksyloitumisen vaikutusta, insuliiniresistenssiin ja alentuneeseen glukoosinsietoon terveillä pikkukeskosena syntyneillä aikuisilla sekä ylipainoisilla nuorilla aikuisilla. Lisäksi tutkimuksissa määriteltiin seerumin osteokalsiinin ja karboksyloidun osteokalsiinin sekä virtsan osteokalsiinin normaaliarvot lapsilla ja tutkittiin, mitkä tekijät terveillä lapsilla vaikuttavat osteokalsiinin pitoisuuksiin.

Glukoositasapainoa kuvastavien merkkiaineiden ja luuston osteokalsiinin säätelyn välistä yhteyttä ei pystytty osoittamaan tutkituissa kohorteissa. Myöskään glukoosirasituksessa yhteyttä ei voitu todeta minkään aikapisteen osalta. Energia-aineenvaihdunnan muutokset vaikuttivat luun muodostuksen keskeisiin merkkiaineisiin mutta insuliinin merkitys niitä säätelevänä tekijänä ei saanut vahvistusta. Päinvastoin kuin eläinmalleissa, seerumin osteokalsiinilla voi olla yhteys pitkäaikaisen glukoositasapainon säätelyyn kun taas glukoosirasituksen yhteydessä akuuttiin glukoosiaineenvaihdunnan säätelyyn vaikuttavat todennäköisesti muut välittäjämekanismit.

Lapsuudessa ja nuoruusiässä ikä, pituus ja paino, sekä lisäkilpirauhashormoni ja puberteetti ovat itsenäisiä seerumin ja virtsan osteokalsiinipitoisuuksia määritteleviä tekijöitä. Verenkierrossa tavattava osteokalsiini kuvastaa enemmän puberteetin aikaista kasvua kuin hetkittäistä luuston mineralisoitumista. Siksi sen kuten myös muiden luuston merkkiaineiden merkitys luuston terveydentilan arvioinnissa terveillä yksilöillä on rajallinen.

Tämän väitöskirjahankkeen tulokset eivät tue väitteitä osteokalsiinin erityisestä merkitsevyydestä energia-aineenvaihdunnan säätelijänä. Aikuisilla osteokalsiini on silti edelleen hyvä luuston aineenvaihdunnan merkkiaine erityisesti arvioitaessa heikentyneeseen luustoon kohdistuvan lääkityksen hoitovastetta.

6. Introduction

Homeostasis (originating from Greek: ὅμοιος homœos, "similar" and στάσις stasis, "standing still") as defined already by Claude Bernard in 1865, is the *property of a system in which variables are regulated so that internal conditions remain stable and relatively constant*. Regulation of one organ or a system occurs through function-limiting feedback, which has also been recognised as one of the cardinal rules of endocrinology while it applies to most physiological functions- a principle positing that different organs exert opposite, regulatory effects on the same function or organ[1].

Historically, bone has not been acknowledged to exert any such endocrine functions. Implementation of this endocrine concept in the recent studies exploring the effect of bone on glucose and energy metabolism has modified our understanding and view of an osteoblast-derived molecule, osteocalcin (OC) and its potential role in glucose and energy homeostasis.

Already in early 1990's, serum OC was established as a marker of both high bone formation but also accelerated bone turnover[2]. As OC is being specifically expressed by osteoblasts, most of the historical studies have focused on the characterisation of OC as a predictor of fractures and solely as a component of bone metabolism. Despite many *in vitro* and *in vivo* studies over the past decades, the specific function of OC remains perplexing. In addition, its presumed receptor yet remains to be identified. Only recently, the role of OC as an endocrine factor exerting a significant control on energy metabolism has been suggested. However, the presented evidence were mostly, if not solely, described in mice, the animal, which represents the renaissance of the study of physiology through genetics. While the applicability of these results in a more complex organism such as humans, is questionable, especially viewed from the perspective of the field of endocrinology and concept of homeostasis.

As the evidence regarding the suggested link between OC and energy metabolism in humans was scant or equivocal, confounded by the selection of populations with multiple co-morbidities influencing their metabolic homeostasis, the concept of bi-directional regulatory role of OC remains controversial, debated and undetermined. Thus, the question remains- if the skeleton and the metabolically active bone tissue are influenced by the organs, which are not classically affected by it and furthermore, whether the skeleton influences them in return instead.

7. Review of literature

7.1 Bone structure and anatomical architecture

Bone is a dynamic organ containing even a cell type, osteoclast, which is curiously targeting to destroy itself, the host[3]. However, the paradigm of bone biology is relatively new as only since 1950's the subsidiary role of bone as a participant in haematopoiesis or calcium homeostasis additional to its structural functions related to mobility, posture or protection of our inner organs and bone marrow, was stipulated.

Anatomically bones can be divided into two main categories, which are produced via two distinct processes during foetal development and longitudinal growth[4]. Intra-membranous ossification, growth of the primary or secondary ossification centres, prevails in flat bones such as the skull bones or clavicle. The process occurs in condensations with embryonic connective tissue by proliferation and differentiation of mesenchymal progenitor cells directly into bone forming cells[4]. On the other hand, long limb bones, with tubular structure, such as the femur or tibia, are dependent on cartilage template and endochondral ossification, which occurs in the growth plates located between metaphysis and epiphyses[5]. The epiphyseal growth plate has a crucial role in bone growth and two opposing processes determine its thickness: 1) chondrocyte proliferation and hypertrophy, and 2) chondrocyte apoptosis and vascular invasion of the growth plate, followed by conversion into primary bone spongiosa[6].

The external parts and the diaphysis of the bone, as most of our skeleton (up to 80%) comprise predominantly of cortical bone[5]. Structurally it consists of dense layers of calcified tissue arranged as concentric lamellae forming osteons with a Haversian canal in the middle. In turn, vertebral columns and metaphyseal areas, parallel with the epiphysis of long bones, are macroscopically made of trabecular bone. Trabecular, or cancellous bone, is a less dense network of calcified trabeculae which represents the greatest surface of all bone structures and the metabolically active tissue maintaining the mineral homeostasis. Both cortical and trabecular bone are composed by the same bone cells and extracellular matrix with its inorganic and organic components; minerals, collagen fibres such as Type I collagen, and other non-collagen proteins[7].

7.2 Normal bone turnover and metabolism

Bone as a tissue has a well-established and regulated metabolic function beyond its structural and architectural functionality as it participates in homeostasis by storing and releasing two essential minerals, calcium and phosphate[7,8]. Up to 99% of the body calcium is stored in bone, bound to the bone matrix in the form of hydroxyapatite crystals (HAP)[9]. These small, soluble crystals provide mechanical rigidity and load bearing strength to the organic bone matrix but also an accessible reservoir for the minerals and other ions such as phosphate[10]. Phosphorus is an essential element of bone and plays an important role in multiple biological processes[11]. The homeostasis of phosphate is determined by modulation of intestinal uptake of dietary phosphate, renal phosphate reabsorption and excretion, and the exchange of phosphate between extracellular and bone storage pools[12]. Maintenance of adequate inorganic phosphorus levels is essential for bone matrix mineralisation[13], which is required also for vascular invasion of the growth plate cartilage, and is delayed or prevented by calcium or phosphorus deficiencies[6].

The composite design of bone defines its strength, which is depending on the structural and material components. In order to maintain its structural integrity and fulfil its metabolic function the skeleton must also renew bone through a process called remodeling.

7.2.1 Remodeling

The bone tissue is being dynamically and continuously remodelled by the coupled processes of bone resorption by osteoclasts and bone formation by osteoblasts[14]. Bone remodeling assists in adjusting the bone architecture to meet altering mechanical needs and it helps to repair microdamages in bone matrix and thus preventing the accumulation of old bone. It consists of a series of sequential, highly regulated steps; removal of mineralised bone tissue by mature osteoclasts, which leaves a resorptive cavity to be filled by the migrating osteoblast precursors, which later differentiate into mature osteoblasts. Osteocytes, the most abundant and long-lived cells within the bone representing the terminal differentiation stage of osteoblasts, regulate both remodeling and mineralisation processes while being embedded in the mineralised bone matrix consisting of collagen[15].

The bone remodeling cycle begins with activation mediated by cells of the osteoblast lineage but it may involve the osteocytes, the lining cells, and the preosteoblasts located in the bone marrow. The cells participating in remodeling undergo structural changes in their shape, secrete enzymes, which digest and degrade proteins on the bone surface and express a 317 amino acid peptide called receptor activator of NF-kappa B ligand (RANKL). The interaction of RANKL with its receptor on osteoclast precursors called RANK results in activation, differentiation, and fusion of hematopoietic cells of the osteoclast lineage. This RANKL/RANK interaction starts the process of resorption but also confirms the role of RANKL in coupled bone resorption and formation. Furthermore, it also prolongs osteoclast survival by suppressing apoptosis[16] while its own activity is blocked by osteoprotegerin (OPG), which acts as a soluble receptor acting as an antagonist, a decoy. OPG is primarily produced by the cells of osteoblast lineage and it regulates bone resorption by inhibiting the final osteoclast differentiation and activation and by inducing their apoptosis[16].

The entire remodeling process takes place in a coordinated fashion and in distinct places as the bone formation only takes place at sites where bone has been recently resorbed[17]. A complete remodeling cycle takes 3-4 months, 10% of all bone tissue at any point in time is being remodeled, every day and 24/7, and up to one quarter of trabecular bone is annually being replaced in adults[7]. Collectively these continued remodeling cycles in an adult skeleton lead to replacement of bone tissue every 10 years.

During a lifetime, there are several diverse phases of bone turnover with either accelerated bone building or slow decline in bone mass or bone mineral density (BMD). Growth spurts during periods in childhood or puberty lead towards attainment of the maximum bone mass, peak bone mass, in a healthy adult full-grown skeleton around the age of 20 years[18]. After having reached the peak bone mass and especially after the 40th year of life the resorption overtakes the speed of bone building and the skeleton starts to gradually lose bone[19],[20].

Skeletal remodeling can be triggered by multiple factors: mechanical forces, bone micro-damage or by hormonal, calcium-regulating or sex hormone, even leptin, responses to change in mineral supply. Additional factors, which influence remodeling, are listed in Text box 1[21]:

Text box 1: Factors influencing remodeling

- *Circadian rhythm*
- *Diet*
- *Physical activity*
- *Season*
- *Pregnancy*
- *Stage of the menstrual cycle*
- *Age*
- *Sex*
- *Race*
- *Presence of fractures*
- *Concomitant disease(s)*
- *Concomitant medication(s)*

7.2.2 Modeling

During childhood and adolescence, bones are sculpted by modeling, which allows for the formation of new bone at one site and the removal of old bone from another site within the same bone[22]. These independent processes thus occur on different surfaces of the bone (vs. coupled remodeling process)[23],[24]. This allows individual bones to grow in size and length, to shift in space or reshape, resulting also in a net increase of bone mass. During childhood, bones grow because resorption occurs inside the bone while formation of new bone occurs on its outer, periosteal surface. At puberty, the bones get thicker because formation can occur on both the peri- and endo-osteal (inner) surfaces.

In a developing foetus, a total of 80% of foetal bone mineralisation occurs during the last trimester of a normal pregnancy[25]. Acquisition of bone mass later in childhood results from bone formation without preceding resorption at a particular site. However, also bone resorption takes place during this time even if the rapidly occurring bone formation by far exceeds the rate of resorption[26]. The lengthening and widening of the bones and accrual of higher BMD leads to a 30-fold increase in bone mass from infancy to adulthood[27]. There appears to be no relationship between the duration of puberty and accrual of peak bone mass[28].

7.2.3 Hormonal regulation of bone turnover

Calcium and phosphate regulating hormones

Calcium and phosphate regulating hormones play a vital role in healthy bone metabolism (Figure 1). Parathyroid hormone (PTH) maintains the level of calcium and stimulates both resorption and formation of bone[29]. Calcitriol, the hormonally active metabolite of vitamin D (also called 1,25-dihydroxycholecalciferol or 1,25-dihydroxyvitamin D3, i.e. 1,25 $(OH)_2D_3$), stimulates sufficient intestinal absorption of calcium and phosphorus while it furthermore also directly affects bone metabolism[29]. The third calcium-regulating hormone, calcitonin, counteracts bone resorption and may protect against excessively high levels of calcium, hypercalcaemia. Fibroblast Growth Factor 23 (FGF-23), a counter-regulatory hormone for 1,25 $(OH)_2D_3$, reduces serum PTH levels via signal transduction in the parathyroid gland and contributes to a prolonged negative feedback loop additionally involving bone and kidney[30,31].

PTH is produced by four small parathyroid glands adjacent to the thyroid gland. These glands are sensitive to rapid changes in calcium concentration: when concentration of calcium decreases even marginally, the secretion of PTH immediately increases. In order to conserve calcium, PTH increases proximal tubular expression of 25(OH)D 1α-hydroxylase, resulting in increased production of $1,25(OH)_2D_3$ and, consequently, increased calcium absorption in the gut. PTH similarly enhances calcium reabsorption in the distal convoluted tubule[32]. PTH also acts directly on the bone to increase movement of calcium from bone to extracellular fluid by increasing osteoclastic bone resorption. Excessive production of PTH, hyperparathyroidism, usually induced due to a small tumour of the parathyroid glands, can lead to bone loss[29]. PTH hormonally regulates also the plasma phosphate concentrations, causing an immediate reduction in phosphate levels via action on handling of phosphate in proximal tubule[33]. Chronic elevated PTH concentrations induce a decrease in serum phosphate, which in certain disease conditions may lead to rickets and osteomalacia.

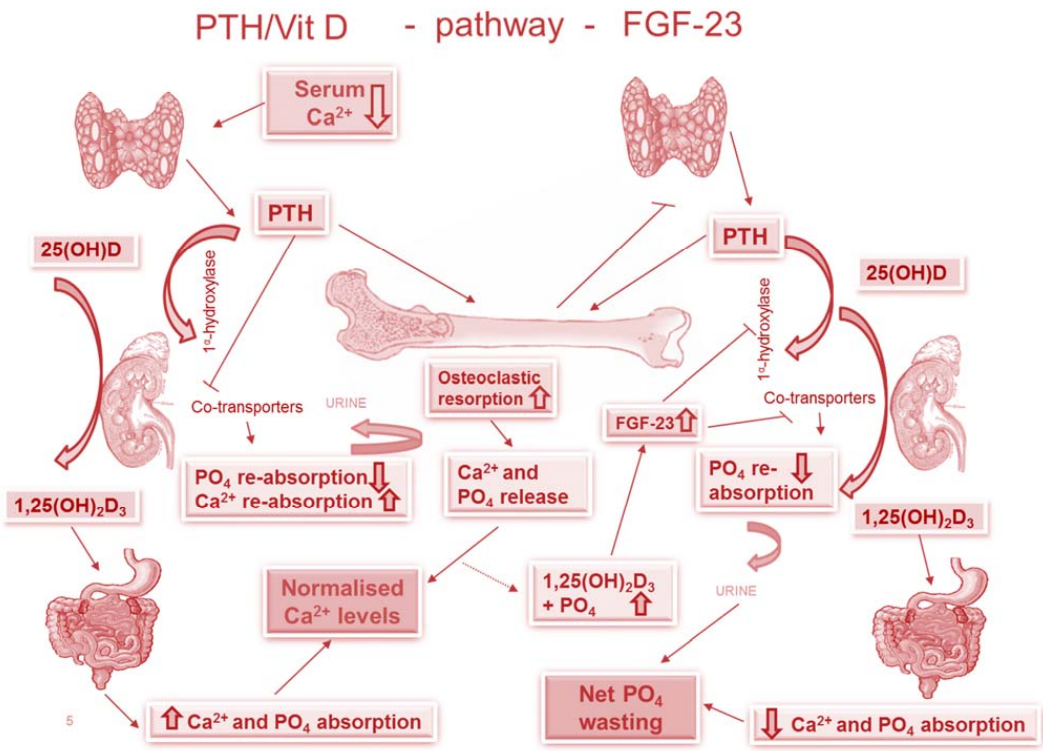
PTH can exert both anabolic and catabolic effect on bone, stimulation both bone formation as well as resorption. The effect of therapeutic, exogenously administrated PTH on bone is dependent on the mode of administration: when small amounts of PTH are administered intermittently, bone formation predominates and the bone strength increases[34]. PTH and $1,25(OH)_2D_3$ also regulated remodelling by stimulating RANKL secretion[35,36].

The second hormone related to PTH, parathyroid hormone-related protein (**PTHrP**), typically regulates cartilage and bone development in the foetus[37] while over-production might indicate malignancy. PTHrP acts similar to PTH, i.e. causing excessive bone breakdown and malignant hypercalcaemia[38].

FGF-23, a phosphatonin regulating the phosphate homeostasis, is a 251-amino acid glycoprotein, which adjusts the physiological response to dietary phosphate load. Increased oral phosphate intake results in increased secretion and higher serum levels of FGF-23, leading to decreased absorption of phosphorus from the gut. Its N and C terminals are participating in the hormone's activity; N-terminal peptide binds to its receptor in the tissues and its C-terminal binds to Klotho[39]. FGF-23 is predominantly expressed in bone, in osteocytes[40], but it is also expressed in a variety of human and mouse tissues, including brain, thymus, small intestine, heart, lung, liver, kidney, thyroid/parathyroid, lymph node, skeletal muscle, spleen, skin, stomach, and testis[41,42,43,44]. FGF-23 causes a decrease in phosphate reabsorption by downregulating the sodium phosphate co-transporters in the proximal tubule, leading to phosphaturia and hypophosphatemia. It lowers serum levels of active Vitamin D by diminishing the production of calcitriol and increasing its catabolism by inhibiting the activity of renal 1α-hydroxylase and stimulating

that of 24-hydroxylase, respectively[45].The effect of FGF-23 on proximal tubular phosphate reabsorption, which requires hours or even days, is independent of PTH and calcitriol[46].

Figure 1: The role of calcium and phosphate (PO_4) regulating hormones on bone- the PTH/vitamin D or FGF-23 pathways. The parathyroid gland detects changes in the level of Ca^{2+} in blood, leading to secretion of PTH. A decrease in circulating calcium stimulates the parathyroid gland to produce and release PTH. PTH increases calcium levels in a pleiotropic fashion by stimulating osteoclastic bone resorption to release calcium and phosphate, calcium reabsorption and phosphate excretion in the renal distal convoluted tubule by downregulating the co-transporters and production of $1,25(OH)_2D_3$ by 1α-hydroxylase in the kidney, which, in turn, increases intestinal calcium and phosphate absorption. The kidney is the principle physiological target, where FGF-23 signalling acts to promote PO4 excretion by downregulating co-transporters and inhibiting $1,25(OH)_2D_3$ production, thus preventing Vitamin D-mediated PO_4 absorption in the gut. Serum levels of FGF-23 increase in response to increased serum PO_4 and 1,25(OH)2D, and furthermore, FGF-23 inhibits the production of PTH.



Calcitonin, also known as thyrocalcitonin, is a 32-amino acid polypeptide produced by parafollicular C-cells of the thyroid gland[47]. Calcitonin counteracts the effect of PTH and it can offset bone resorption by inactivating osteoclasts[48]. This effect may be relatively transient in adults. Calcitonin may be more important for maintaining bone development and normal blood calcium levels in early life. Excesses or deficiencies of calcitonin in adults do not cause impediments in maintaining blood calcium concentration or the strength of the bone, while during periods of mobilisation of calcium, such as pregnancy or lactation, calcitonin protects against calcium loss[49]. Calcitonin has also been clinically utilised as an anti-resorptive therapy, even if with relatively modest effectiveness, in postmenopausal osteoporosis[50].

Calcitriol, 1,25-$(OH)_2$ vitamin D, is the metabolically active form of Vitamin D. It is produced stepwise from 7-dehydroxycholesterol after exposure to the ultraviolet B radiation on the skin which induces the production of vitamin D_3, cholecalciferol. D_3, which can also be obtained from various nutrients such as fatty fish or fortified dairy products[51], is metabolised in the liver into 25-hydroxyvitamin D (25-OHD or calcidiol) prior to its conversion into calcitriol in the kidneys. Presence of calcitriol is essential for skeletal growth in infancy and early childhood as it increases supply of essential minerals for bone by increasing intestinal absorption of calcium and phosphorus. Deleterious effect on, or impairment of bone metabolism or bone growth, have been described in subjects with low vitamin D levels or in infants born to mothers with low maternal vitamin D status during pregnancy[52].

Many individuals need vitamin D supplementation due to inadequate levels derived from exposure to the sun. Historically, a need for supplementation has been increasingly rising as people began to live indoors, wear clothes, and move further towards northern latitudes where the Sun's rays are increasingly filtered during the winter months, leading to insufficient production of vitamin D in the skin. Deficiency of Vitamin D leads to a disease of defective mineralisation, called rickets in children and osteomalacia in adults. These conditions can result in bone pain, bowing and deformities of the legs, and fractures. Treatment with vitamin D can restore calcium supplies and reduce bone loss[53].

Sex hormones

Besides the calcium-regulating hormones, also sex hormones are essential for regulation of growth of the skeleton and maintenance of the bone mass and its strength. Oestrogen and testosterone both have effects on bone in both sexes[54] and gonadal failure or dysfunction lead to lower bone mass[55]. The oestrogen produced in children and early puberty induces bone growth while the high concentration at the end of puberty stops further growth in height by closing the cartilage plates at the ends of long limb bones[56].

Oestrogen acts on both osteoclasts and osteoblasts at all stages in life by inhibiting bone resorption[57]. Consequently, the marked decrease in oestrogen at menopause or withdrawal of oestrogen is associated with increased bone remodeling, increased depth of resorption sites and increase in osteoclastogenesis, leading to rapid net bone loss[58]. Oestrogen may also act indirectly by influencing the extra-skeletal calcium homeostasis and systemic hormones or by modulating the activity of various cytokines or growth hormone[59].

Testosterone is the main circulating androgen in males and it is important for skeletal growth and maintenance because of its direct and indirect effects on bone: testosterone inhibits bone resorption but it also affects bone formation through stimulation of muscle growth, which in turn puts greater stress on the bone, leading to bone formation. In turn, hypogonadal men have been observed to present with an accelerated rate of bone turnover and increased fracture risk[60]. Testosterone is also a source of oestrogen

in the male body, including bones, as it is converted into oestradiol via aromatisation. This source of oestrogen is important for the male bones as well as for women and both animal and human studies suggest that testosterone has a dual mode of action on different bone surfaces with involvement of both androgen and oestrogen receptor[61]. In fact, older men have even higher levels of circulating oestrogen than postmenopausal women[60].

Other Important Hormones

Growth hormone (GH), also known as somatotropin and secreted from the pituitary gland, is an important regulator of skeletal growth[62]. It acts by stimulating the production of another hormone called **insulin-like growth factor-1** (IGF-1), which in turn is released into circulation from the liver. GH also regulates the local production of IGF-1 in other tissues, in particular in bone, while GH may also directly affect the bone[62]. GH is essential for growth and it accelerates skeletal growth at puberty. Decreased production of GH and IGF-1 with age may be responsible for the inability of older individuals to form bone rapidly or to replace bone lost by resorption[63]. The GH/IGF-1 system stimulates both the bone-resorbing and bone-forming cells, but the dominant effect is on bone formation, thus resulting in an increase in bone mass[64].

Thyroid hormones increase the energy production of all body cells, including bone cells. These hormones increase the rates of both bone formation and resorption[65]. Thyroid hormone deficiency can induce impaired growth in children, while excessive amounts of it can stimulate resorption and weaken the skeleton[66]. The pituitary hormone that controls the thyroid gland, thyreotropin or thyroid-stimulating hormone (TSH), may also have direct effects on bone[67].

Cortisol, the major hormone of the adrenal gland, is a critical regulator of metabolism and is important to the body's ability to respond to even extreme stress and injury[68]. It has complex effects on the skeleton, as small quantities are necessary for normal bone development while larger amounts block bone growth[69]. Synthetic forms of cortisol, glucocorticoids, are used to treat many inflammatory diseases such as asthma and arthritis. They can cause bone loss due to both decreased bone formation and increased bone resorption, both of which lead to an elevated risk of fractures[70]

Moreover, there are other circulating hormones that affect the skeleton as well. **Insulin** is important for bone growth, and the response to other bone growth stimulating factors has been demonstrated to be impaired in individuals with insulin deficiency[71],[72]. A recently discovered adipocyte-derived hormone, **leptin**, has also been shown to have effects on bone[73],[74]. The documented, but also, speculated bi-directional effect of these hormones on bone will be discussed later.

7.3 Monitoring bone turnover

Different bone markers and their serum or urine levels reflect different steps of the constantly ongoing, coupled bone remodeling (see also section 7.2.1)[75]. The remodeling cycle consists of three consecutive phases: 1) resorption, during which osteoclasts digest old bone; 2) reversal, when mononuclear cells appear on the bone surface; and 3) formation, when osteoblasts lay down new bone until the resorbed bone has been completely replaced. Resorption probably continues for about 2 weeks, the reversal phase may last up to a few weeks, while formation can continue for 4 months until the new bone structural unit is completely created. Therefore analysis of several bone markers from the same individuals may provide information about different aspects on remodeling activity of the skeleton, its local or systemic regulation and status of calcium and/or phosphate homeostasis.

Biochemical bone turnover markers (BTMs) reflect enzymatic activity of the bone cells, excess products from the formation or fragments released during the degradation of matrix components[76, 77, 78] (Figure 2). Circulating proteins or their fragments detectable in serum are either derived from the bone resorption process when proteins embedded in the bone matrix are being released, formed during proteolytic activity in the blood or reflect the de-novo biosynthesis of the intact proteins while the fragments accumulated in the urine are products of catabolism via renal filtration and diverse degradation cascades. Circulating BTMs also demonstrate dependence on circadian rhythm, characterised by a decrease during the morning hours until noon[21]. Other factors influencing remodelling (Text box 1) also affect the levels of BTMs and should be taken into account when interpreting the results and potential sources of different and potentially changing levels of variable BTMs.

BTMs are, as per their definition specific bone-derived chemical compounds, which a) can be detected in serum, plasma, or urine, b) preferably reflect different dimensions of bone turnover, i.e. resorption, formation or combinations of both, and c) reflect the bone metabolic activity in total body at a given time-point[79].

BTMs may reflect the dynamics (Text box 2) and functions (Text box 3) of many diverse compartments of bone [80, 81].

Text box 2: BTMs per bone compartments

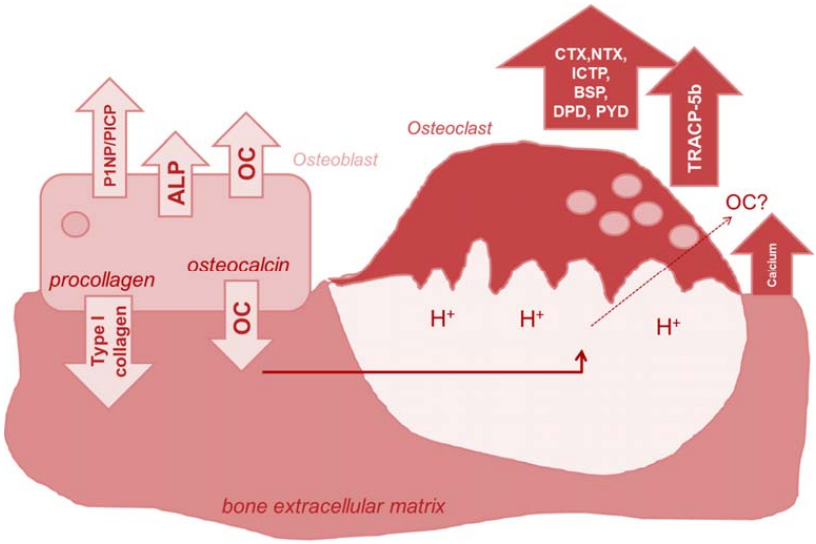
- *a part of the matrix (OC, osteonectin, and osteopontin)*
- *precursors or degradation products of the matrix (pro-collagen or cross-links of collagen)*
- *matrix enzymes (ALP, tartrate resistant acid phosphatase (TRAP-5b))*
- *signaling substances such as OC or sclerostin*

Text box 3: Different functions or roles of BTMs

- *mineralised matrix (hydroxyapatite, i.e. calcium and phosphate)*
- *non-mineralised matrix (collagen, OC, matrix metalloproteinase enzymes, osteopontin, osteonectin)*
- *cellular components of the matrix (osteoclasts, osteoblasts, and osteocytes)*

Some compounds may exert several roles; OC with its alleged hormonal properties is an established part of the non-mineralised matrix supporting bone remodeling but also maturation of the mineral phase[2]. Another example is ALP, which is a matrix enzyme involved in initiation of bone mineralisation but also a marker of cellular, osteoblast, function[83]. Many of the markers are also associated with different steps in either bone formation or resorption; when the rate of bone metabolism is increased both resorption and formation markers will increase to indicate increased bone turnover. For clinical assessment and decision-making, monitoring of at least two different markers reflecting different phases or steps of bone turnover is recommended.

Figure 2: Key bone turnover markers indicating bone formation (pink) and bone resorption (dark red)[80],[81],[82].



Adapted based on reviews and text book chapters by Seibel (2000); Seibel et al (2002) and Hammett-Stabler (2004). H+ indicates hydrogen ions, other abbreviations used for bone turnover markers are given in section 7.3.1. and 7.3.2.

BTMs may be utilised as basis for clinical decisions when monitoring a benign or malignant bone condition, (desired rapid) effect of a medication[83] but not for diagnosis of bone-related diseases or conditions. The use of BTMs could only be considered clinically meaningful if the change on individual patient level for a given BTM were greater than expected from normal variability of the marker. In general, the least significant change is therefore the minimum change between two successive results in an individual that would imply a real biological response. However, in pivotal clinical trials changes on population level reflect efficacy while on individual level a lack of change in BTMs might also be a sign of reduced adherence to therapy or e.g. limited absorption of orally administered anti-resorptive treatment regimens[84].

7.3.1 Bone resorption markers

BTMs describing degradation of mature type I collagen, such as pyridinoline (PYD) and deoxypyridinoline (DPD) crosslinks, are almost solely derived from high turnover bone tissue and not affected by degradation of newly produced collagen[85]. The telopeptide regions of type I collagen can also be assessed with immunoassays by recognising its cross-link free telopeptides[81] such as the dynamic C-terminal telopeptide (CTX), its extended fragments produced by cathepsin K (ICTP[86]) or the N-terminal variant (NTX), which is often being assessed from urine. The iso-form 5b of the tartrate-resistant acid phosphatase family (TRAP-5b) has been identified as a BTM, which specifically reflects the rate of bone resorption and osteoclast activity[87]. Additionally bone resorption can be assessed by more seldom utilised BTMs such as e.g. hydroxyproline or bone sial protein (BSP).

7.3.2 Bone formation markers

Presence of pro-peptides of type I collagen, or rather its C- (PICP) and N (PINP or P1NP)-terminal ends reflect cleaving of peptide processing and formation of collagen. Even if type I collagen is also a component of many other tissues, the collagen turnover is more pronounced in bone vs. non-skeletal tissues and thus elevated levels of PICP and PINP are generally accepted to reflect increased bone formation[81]. While most of the commonly assessed BTMs are cleavage products of collagen, bone – specific alkaline phosphatase (BSAP), the activity of this membrane-bound enzyme can also be used as a marker of bone formation. BSAP is expressed in osteoblasts and it is released into circulation during bone formation process. The levels of total alkaline phosphatase represents a pool of isoforms but the bone-specific BSP as a highly specific and affordable BTM[80].

The characteristics and biological functionality of OC, a matrix protein used for assessment of bone formation among many other dimensions of bone turnover, will be discussed later in this review.

7.3.3 Establishing paediatric reference ranges

Variation in BTMs increases with puberty, demonstrating correlation with growth velocity in healthy children[88,89,90] and in those with pathologically increased growth velocity[91]. Consequently, children and adolescents have elevated concentrations of BTMs due to high skeletal growth and rapid bone turnover compared with adults[92]. The highest levels of bone markers have been described in infants and children during the first four years of life; thereafter levels decrease slightly and remain relatively stable until peaking again at puberty[93].

The timing of puberty is crucial for final bone mass accrual as 25% increase in lumbar spine volumetric BMD occurs during puberty and an increase of 25% in lumbar spine volume[94] is being built up during pubertal growth spurt. By the age of 18, a total of almost 90% of the skeletal mass is being attained[95] even if the process is individual and influenced by many physiological and pathological processes and factors, both in adults and in paediatric populations[96,97], as listed in Text box 4**Error! Reference source not found.**.

Text box 4: Factors influencing bone mass accrual.

- *Genetics and race*
- *Gender*
- *Prematurity*
- *Endocrine and mechanistic factors*
- *(Mal)nutrition*
- *Pharmacological factors*

Consequently, paediatric normative values are vital for differentiating between normal and abnormal bone turnover, investigating skeletal diseases in children or monitoring responses to bone treatment. Reliable assessment of paediatric reference data for BTMs requires a representative sample population of healthy children and adolescents within a defined geographical area. Potential geographical factors influencing reference values are listed in Text box 5:

Text box 5: Geographical factors influencing reference values

- *Vitamin D (in)sufficiency (depending on sunlight)*
- *Dietary habits such as milk intake*
- *National/regional recommendations for supplementation of minerals or vitamins*
- *Exercise habits/programs defined by national school exercise guidelines or recommendations*
- *Religious factors such as veiling*

Hence, every geographical area should ideally have access to local reference values, which provide guidance for interpretation of the BTM results, taking into account the locally most influencing factors.

7.4 Glucose homeostasis and bone

7.4.1 Normal glucose tolerance

The foundation of the human energy metabolism, parallel to any living organism, is related to the immediate need for sufficient supply of glucose to the target tissues via firmly regulated pathways. Glucose homeostasis in humans is regulated by a feedback loop, which includes insulin-sensitive tissues such as pancreatic islet cells; mostly glucagon-producing alpha and insulin-producing beta cells; liver, muscle and adipose tissue[98]. The plasma concentration of glucose is a product of the rate of the glucose entering the circulation balanced by the rate of glucose disposal from the circulation[99]. In a fasting state up to 80% of the glucose released into the circulation originates from the liver while kidneys are responsible to contribute to the remaining 20% through renal gluconeogenesis[100].

Digestion of food in the intestines converts the compacted storage carbohydrates, starch and glycogen, to glucose. Pancreatic β-cells express glucose transporters, which permit rapid glucose uptake regardless of the extracellular glucose concentration[101]. Insulin, a 33-amino acid long peptide is first synthesised as preproinsulin and processed to proinsulin. Thereafter, proinsulin is converted to insulin and C-peptide, stored in secretary granules and awaiting release via fusion of the secretory granules with plasma membrane on demand. Insulin is secreted primarily in response to glucose, while other nutrients such as free fatty acids and amino acids can augment glucose-induced insulin secretion[102]. Glucose-stimulated basal insulin secretion, which secures the insulin necessary for basic energy needs and regulation of hepatic glucose production, occurs in fasting state while stimulated (prandial, bolus) insulin secretion is induced by increasing concentrations of glucose related to a meal or an OGTT[103],[104]. In normoglycaemic individuals but also, when mimicking natural insulin secretion in insulin-insufficient individuals, approximately half of the total daily insulin is secreted or to be replaced as basal insulin, suppressing lipolysis, proteolysis, and glycogenolysis[105].

During stimulated insulin-secretion, a rapid "first phase" of insulin secretion promotes peripheral utilisation of the prandial nutrient load, suppresses hepatic glucose production, and limits postprandial glucose elevation[106]. First-phase insulin secretion begins within 2 minutes of nutrient ingestion and continues for 10 to 15 minutes. This initial secretion is followed by the second phase of prandial insulin secretion, continuing until a normoglycaemic state is again restored. In patients with impaired glucose tolerance (IGT) or in the early stages of type 2 diabetes (T2DM), first-phase insulin release is almost invariably lost despite the enhancement of second-phase secretion[107].

A negative relationship has been demonstrated between the principal, first phase insulin release and initial glucose increment after commencement of a glucose infusion[108]. An early surge of insulin secretion could potentially restrict the rise in blood glucose levels but still, the greater the insulin surge, the more prolonged seems the effect on glucose homeostasis. The effects of an acute first-phase insulin secretion has been shown to significantly affect consequent glucose tolerance by warranting more physiological plasma glucose levels[109].

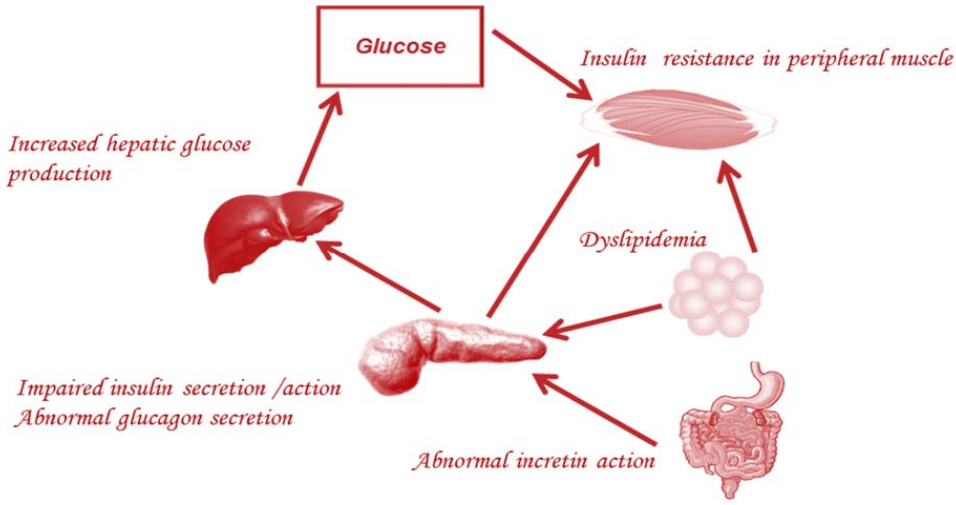
Insulin suppresses hepatic glucose output while it stimulates the uptake of glucose, amino acids and fatty acids in the muscles and adipose tissue[98]. The amount of secreted insulin is determined by the insulin sensitivity, the systemic responsiveness of the tissues to insulin but also the often progressive β-cell failure, or rather, a loss of β- cell function than complete loss of β- mass, which critically impairs the ability of the pancreas to respond to the glucose load [98],[110].

7.4.2 Increasing insulin resistance, hyperinsulinemia and progression to Type 2 Diabetes

In the presence of insulin resistance, the target cells fail to respond to the insulin. Therefore, in response to sustained or prolonged elevated plasma glucose levels the pancreatic beta cells increase production of insulin in order to maintain normal glucose level[98]. This leads to hyperinsulinemia until the β- cells fail especially to respond with the rapid first-phase insulin release and the total amount of secreted insulin declines. Chronic, physiological hyperinsulinemia leads to the development of non-glycogen pathway - dependent insulin resistance, i.e. hyperinsulinemia might not only be a compensatory response to insulin resistance but also become a self-perpetuating cause of the defect in insulin action[111]. The progressive decline in beta cell function and insulin action in the presence of insulin resistance gradually leads to hyperglycaemia and T2DM.

Many factors such as reduced glucose utilisation by peripheral tissues (muscle and adipose tissues) during the absorptive state may result in increase in glucose concentration (Figure 3). Hepatic insulin resistance at may lead to insufficient suppression of (especially nocturnal) glucose production and output as well as an increase in prandial glucose excursion(s). Yet, other potential influencing factors regulating the hepatic glucose release, such as inadequate suppression of prandial plasma glucagon concentration, persistence of elevated prandial plasma free triglyceride (TG) levels, and changes in gastric emptying or motility due to abnormal incretin effect in addition to its role in glucose homeostasis, play a central role[112].

Figure 3: Pathophysiological processes contributing to increased hyperglycaemia in the presence of insulin resistance.



Adapted from Kahn SE, Cooper ME and Del Prato S (2014). Pathophysiology and treatment of Type 2 Diabetes: perspectives on the past, present and future. Lancet 383:1068-1083.

7.4.3 Effect of obesity and impaired glycaemia on bone homeostasis

Obesity, impaired glucose tolerance and T2DM affect bone metabolism. Obesity, with or without glycaemic impairment such as T2DM, may have detrimental effects on bone metabolism through inflammatory cytokines, adipokines, and increased levels of free fatty acids[113]. However, for almost a century, there have been references in the medical literature indicating an increased risk of fractures among patients with diabetes[114]. This clinically relevant increased fracture risk, is not explained by lower BMD (when measured with DXA)[115],[116]. In addition, detection of this elevated risk with regular screening of BTMs is not feasible or theoretically even possible, as no definite extrapolations can be made regarding the ability of BTMs to predict fractures in individuals with hyperglycaemia or diabetes, independent of weight[117].

Patients with T2DM have a generally increased risk of fractures although their overall BMD is not significantly decreased[118]. The rate of bone resorption and formation, as indicated by elevated bone turnover markers, is reduced in subjects with diabetes[119]. On the other hand, improved glycaemic control might stimulate osteoblastic differentiation and enhance bone formation. Markers of bone formation, such as total serum OC, have been shown to be inversely associated with glycaemic control in subjects with T2DM and with the presence of vertebral fractures[120]. Also other sources have demonstrated an increased OC in patients with type 1 diabetes (T1DM)[119].

7.4.4 Effect of low birth weight on glucose and bone metabolism

Increased risk of impaired glucose homeostasis, diabetes and cardiovascular disease later in life is associated with low birth weight[121]. One of the most notable studies by *Hovi et al* assessed the effect of very low birth weight (WLBW, < 1500 g) on glucose and insulin metabolism, glucose tolerance (measured with a 2-hour oral glucose tolerance test; OGTT) and insulin sensitivity (by homeostasis model for insulin resistance, HOMA-IR), serum lipid levels and blood pressure in young adults born with WLBW[122]. This study observed significant differences in markers of glucose homeostasis between the VLBW and control subjects; the VLBW subjects having a 6.7% higher glucose levels, 16.7% higher fasting plasma glucose (FPG) and 40% higher 2-hour insulin concentration after an OGTT. None of the subjects had T2DM but some subjects in both groups had impaired glucose tolerance (IGT). Hyperinsulinemia as a sign of increased insulin resistance and clinically confirmed IGT are generally acknowledged early indicators of impaired regulation of glucose metabolism and reduced peripheral insulin sensitivity[98].

In the same cohort, there were also significant differences in BMD between the VLBW subjects and the controls: the VLBW subjects had a significantly lower lumbar spine (LS), femoral neck (FN) and whole body BMD[123]. The differences in Z scores were up to 0.51 units in the LS and 0.56 in the FN and the differences could only partially be explained by smaller body size or lower exercise intensity. The observed difference remained significant and clinically relevant even after adjustment for several potential confounding factors.

Individuals with VLBW are also known to be predisposed to short final height[124] and as young adults have reduced BMD[125]. This might be due to their smaller size even if the measurements have been adjusted for bone size (by estimation of volumetric bone density)[125]. The association between these clinical characteristics and the impact of VLBW on glucose metabolism and other cardio-metabolic outcomes such as lipid profiles, blood pressure, renal function, urinary albumin, and thyroid function

were investigated in 111 Japanese young adults with VLBW[126]. A total of 36% of the subjects had short stature with a final height below the 10th percentile, 7.2% had IGT or T2DM. Short stature strongly correlated with glucose intolerance (OR 11.1; 95% CI 1.92, 99.7; p=0.006) and the final height was inversely associated with markers of insulin resistance/glucose sensitivity and total/LDL-cholesterol. The associations of final height with insulin sensitivity and lipid profiles remained after adjustment for target height and age at the onset of puberty. The authors concluded that shorter final height was associated with less favourable metabolic profiles in young adults with VLBW, and may be partly mediated by reduced insulin sensitivity. These associations were independent of target height or age at puberty onset.

A limited number of studies have evaluated the markers of bone metabolism in VLBW subjects. Some studies have reported increased OC in subjects born with VLBW[127] while in another study (which did not assess OC) no differences in formation or resorption markers were observed[125].

7.5 The suggested link between osteocalcin and energy metabolism

In general, metabolic diseases are known to influence bone homeostasis[128]. Leptin, an adipocyte-derived hormone, has been previously shown to inhibit bone formation by acting on osteoblasts via central neural pathways[128],[129]. However, based on several recent, experimental evidence[130],[132], OC is believed to play a central role in regulating the cross talk between murine fuel and bone metabolism[128],[129],[130],[132]. These studies have suggested another neuroendocrine circuit linking bone and glucose metabolism and fat mass. These new data imply existence of bi-directional signalling between bone and metabolic /energy homeostasis; OC indicated as a key mediator of this signalling. OC, which exhibits several hormonal characteristics[131], has been indicated to regulate glucose metabolism and fat mass in mice[132]. Yet, its specific function remains indistinct.

7.5.1 Osteocalcin

OC in an osteoblast lineage-derived, Vitamin K-dependent secreted protein, a macromolecule, which is implicated in bone mineralisation as it forms 1-2% of the bone matrix[131] and also in calcium homeostasis. OC exists in the general circulation in fully carboxylated, partially carboxylated and completely uncarboxylated forms but the biological function of these different forms is still not properly mapped. As OC is being specifically expressed by osteoblasts, most of the historical studies have focused on the characterization of OC as a predictor of fractures and solely as a component of bone metabolism. Already in early 1990's serum OC was establish to be a marker of both high bone formation but also high bone turnover[133]. OC has also been shown to correlate with serum testosterone and bone maturation in boys[134].

Previous studies in paediatric populations have demonstrated that serum OC concentration is a sensitive biochemical marker of skeletal development and growth also with changing weight/obesity status[135]. Only recently, the role of osteocalcin as an endocrine factor exerting a significant control on energy metabolism has been suggested.

Serum osteocalcin

Serum OC is one of the very few osteoblast-specific molecules, synthesised in the bone matrix and it has been shown to exhibit several hormonal characteristics; it is a cell-specific molecule, synthesized as a pre-pro-molecule and it is secreted into general circulation[131]. OC undergoes an unusual post-translational modification whereby glutamic acid residues are carboxylated to form γ-carboxyglutamic acid (GLA) residues[131]. This process is Vitamin K dependent[136]. The degree of their carboxylation defines the affinity of the molecule to the skeleton as the formation of GLA residues enables OC and other γ –carboxylated proteins to bind to calcium.

OC's biological function might be related to its structure with GLA residues as OC has been suggested to affect the growth or maturation of the mineral phase, composed by crystalline HAP[137]. This is supported by its first appearance coinciding with the onset of mineralisation in utero[138]. OC levels are also increased in parallel with HAP deposition during skeletal growth and mineral maturation[139].

In mice approximately 10% of the circulating OC present as undercarboxylated OC while in humans it accounts up to 40%[140, 141]. Some studies have though shown that in elderly individuals up to 91% of the total OC is carboxylated[142]. Whereas carboxylated OC (cOC) has a higher affinity for HAP than under/uncarboxylated OC (unOC)[143], the biological role of γ-carboxylation remains partially unclear. For decades, the biological role of OC has been a target of various attempts to identify its role, even during foetal developmental stage. There are limited studies to report the presence of OC in amniotic fluid. *Mimouni et al* reported as early as in 1987 from 15 to 43 weeks of gestation (n=131) the presence of OC, which had the same gel-filtration characteristics as serum OC[144]. In 67% of the samples OC could not be detected, mostly potentially due to their historical RIA method having had an insufficient detection limit (0.1 ng/ml). A total of 95% of the samples had a concentration below 2.4 ng/ml and the authors defined that as a limit for normal levels of OC in amniotic fluid. None of the pregnancies studied lead to birth of a child with a mineral bone disease, and no reports of any metabolic abnormalities were available or studied.

Based on the recent knowledge, advanced technology and development in understanding the role of OC in regulation of energy metabolism, the mapping of OC expression during foetal but also later development might provide us with greater understanding of the biological roles of carboxylated and uncarboxylated forms of OC.

Under/uncarboxylated osteocalcin

A small but detectable and measurable amount of unOC (or Glu-OC; indicating the lack of carboxylation of the glutamic residue) can be detected in the serum[145]. Its presence might indicate that either some OC is being secreted in an incomplete form by the osteoblasts or that OC is enzymatically or otherwise being decarboxylated outside the cells. Only limited number of, mostly non-validated assays, are available for appropriate detection of un OC concentrations and thus most of the studies have estimated the proportion of unOC by simply deducting the amount of cOC from the total OC. Nevertheless, this is not optimal as serum unOC is highly correlated with total OC while the percentage of unOC (%unOC) is not[146]. A few commercially available assays for unOC may overestimate the real OC concentration as the antibody used in the assay recognises the N-terminal fragments of OC[147] (especially if intact), instead of total OC concentrations being appropriately explored.

Urinary osteocalcin

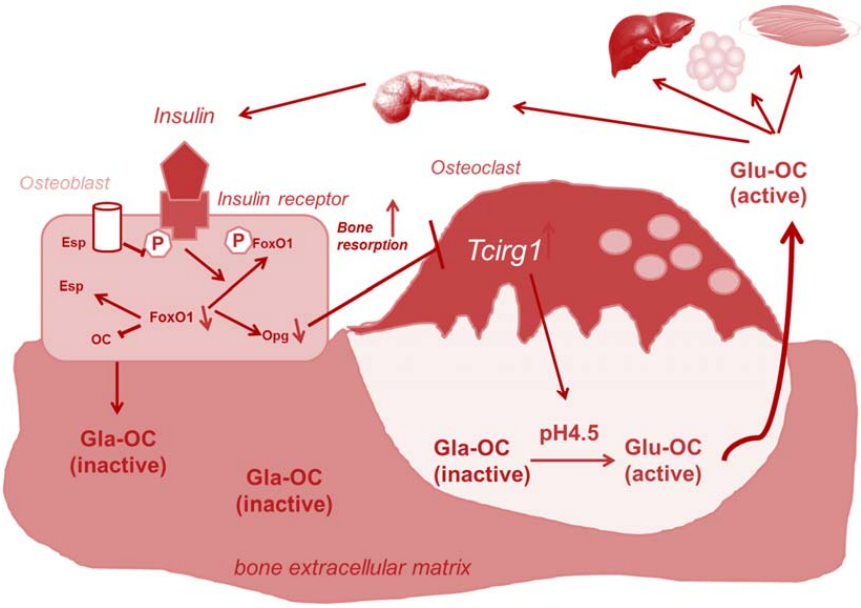
OC is metabolised in urine as measurable mid-molecule fragments (U-midOC). The urinary levels of OC correlate with serum OC concentrations and other serum BTMs in adults[148]. U-midOC molecular fragments, which accumulate in urine, have been used alone in adults as an index of bone turnover, but it not yet been established as a marker of bone turnover in children and adolescents while there are several studies evaluating serum OC in children and adolescence[149],[150],[151]. One study assessed the urinary mid-fragments predicted the catch up growth in prematurely born infants[152].

7.5.2 Preclinical evidence – the foundation of the hypothesis

The group of *Gerard Karsenty* put forward a paradigm-shifting hypothesis (Figure 4) according to which bone contributes in, and even regulates the whole-body physiology, energy metabolism and even fertility[153]. These series of theories were initiated based on findings by *Lee et al* who showed that mice lacking the OC encoding gene (OC -/-) have an abnormal amount of visceral fat, and present with signs of glucose intolerance, insulin resistance and impaired insulin secretion compared to wild-type (wt) mice[132]. On the other hand, administration of recombinant OC in such mice significantly improved glycaemia and increased insulin secretion[130]. Simultaneously, presence of an osteotesticular protein tyrosine phosphatase, OST-PTP, a factor regulating insulin sensitivity via dephosphorylation of the insulin receptor in osteoblasts, was observed in this mouse model. OST-PT is only expressed in osteoblasts and Leydig cells while now claimed to be universally involved in the regulation of carboxylation of OC and its metabolic effects– a claim slightly hampered by the fact that no corresponding analogous molecule could be found in humans. However, another model was suggested to be responsible for the origin of the uncarboxylated form of OC, excluding the previously well-established role of vitamin K in the carboxylation of OC: insulin signaling in osteoblasts. According to this hypothesis, insulin signalling induces a decrease in the expression of OPG, which in turn activates bone resorption and therefore this potentially spontaneous decarboxylation of OC in an acidic environment would promote the effect of unOC on glucose and insulin[154],[155]. Hence, Lee *et al* had for many researchers demonstrated that unOC regulates glucose homeostasis as in their model unOC was alone able to induce expression of adiponectin, insulin and markers of pancreatic islet cell proliferation[132].

In these *ex-vivo* studies by Lee *et al*, insulin secretion was stimulated when isolated pancreatic beta islets from wt mice were co-cultured with wt osteoblasts or in the presence of supernatants from cultured osteoblasts[132]. This hypothesis suggested the presence of an osteoblast-derived circulating factor, which is capable of regulating pancreatic β-cells or even the islet cell function as an entity. In similar co-culture assay of wt osteoblasts with adipocytes, the group demonstrated also an increase in adiponectin expression and action[132]. As per their results, OC and leptin may thus, at least in rodents, contribute to the components of the dysmetabolic phenotype. Increased adiposity, insulin resistance and low bone formation and reduced numbers of osteoblasts were observed also by others studying mice lacking insulin receptors in their osteoblasts but the effect of infused unOC in these mice resulted nevertheless in a modest physiological effect in insulin- and glucose tolerance tests[155],[156].

Figure 4: The hypothesis or speculative model of the role of osteocalcin on energy metabolism via resorption-induced activation of decarboxylated OC, unOC, and its effect on key components of glucose homeostasis.



Adapted from Ferron M et al. Insulin signalling in osteoblasts integrates bone remodeling and energy metabolism. Cell (2010) 142, 296-308 and Karsenty G & Ferron M. The contribution of bone to whole-organism physiology. Nature (2012) 481: 314-320. FoxO1; Forkhead O1 protein which regulates metabolic homeaostasis in response to oxidative stress; Esp, a pseudogene encoding OST-PTP in rodents; Glu-OC and, Gla-OC; different degrees of carboxylation of OC indicating active and inactive forms of OC, respectively.

The effect of these moderate changes in osteoblastic insulin signaling on glucose tolerance was explored in yet another mouse model, which either over- or under- expressed the insulin receptor[157]. No changes were observed in glucose metabolism (vs. wt) when the animals received a normal diet. Yet, when the animals were fed a high-fat diet, insulin resistance was declining in those over-expressing, or exacerbated in those under-expressing the insulin receptor (vs. wt animals on the same high-fat diet)[157]. Simultaneously, parameters exploring the impact of this modification on bone turnover were lower in animals on the high-fat diet with relatively larger reduction in those under-expressing the insulin receptor. Circulating levels of both total OC and unOC decreased in parallel with reduced bone turnover, which was also confirmed by reduction in other BTMs such as CTX and PINP. The researchers gave also daily injections of unOC to all mice, after which glucose tolerance and insulin sensitivity were being

improved[157]. Inconsistently, total OC increased three times that of unOC, suggesting some conflicts or irregularities in their analytical methodology.

On the other hand, the endocrine function of adipose tissue is well characterised[132,158] and studies to establish how adipose tissue and adipocyte-derived endocrine-acting factors affect the skeleton are ongoing. Based on the above-mentioned cell assay and rodent studies, insulin signalling and increased rate of resorption may lead to increase in OC secretion in osteoblasts[154], which in turn increases insulin sensitivity[159] and increase pancreatic insulin production[160]. These preliminary pre-clinical data indicated that the bi-directional regulatory role of the skeleton on glucose and energy homeostasis appears to be mediated by OC[132] but there are only limited clinical data available to confirm this in patients with impaired glucose metabolism or impediments in their metabolic pathways.

One of the key questions thereby is whether bone regulates such physiological functions, which at least primarily seem to have no obvious connection with bone health *per se.* Most hormones exert several functions so why not also OC. According to another exhilarating theory, unOC was also claimed to play a role in male fertility via increased testosterone production in male mice[161,162]. As described above, OST-PTP was identified in testosterone producing Leydig cells and the hormone production was significantly diminished when co-cultured with primary osteoblasts lacking the OC gene[161,162]. Additionally, OC was reported by some, but not confirmed by others, to be a G protein-coupled receptor family C group member 6 member A (GPRC6A) agonist, a receptor on Leydig cells[157,162,163,164]. Nevertheless, although a total OC decreased during use of anti-resorptive medications in human male subjects, no change in circulating testosterone concentrations has been observed[165]. Therefore, the relationship between OC and testosterone may also be reflective of the well-established hormonal relationship between bone and testosterone, related to the role of testosterone in bone formation and resorption[166].

7.5.3 Clinical evidence

There is a wealth of pre-clinical evidence referring to individual findings supporting or mirroring the suggested evidence regarding the bi-directional regulatory pathway after the first conceptual analysis by the group of *Karsenty* were presented. Yet, the clinical evidence is scant and there are no prospective, pivotal clinical studies, which would have addressed this hypothesis.

Post hoc analyses in patients with osteoporosis

Pittas et al were among the first to test and report an inverse association between total serum OC and the markers of metabolic dysfunction (fasting hyperglycaemia, insulin resistance and systemic inflammation) and measures of adiposity (body mass index; BMI and body fat) in a cohort of humans, in this case elderly subjects[167]. In this *post hoc* analysis, 5% of the studied patients had T2DM, 29% IGT and 66% normal FPG. The patients in the highest OC tertile had statistically significantly lower rise in FPG vs. the lower tertiles[167]. Another early report from the Swedish cohort of osteoporotic men (MrOS; a cross-sectional association study in osteoporotic males), indicated that total plasma OC was inversely associated with BMI, fat mass and plasma glucose while it was not significantly associated with height or lean mass[168]. Plasma OC explained 6.3 % and 2.5 % of the variance in plasma glucose levels in all subjects and those without diabetes, respectively. However, this study evaluated again only the total OC levels and not specifically unOC, which had been shown to exert the effects at least in animal studies[132]. Some smaller studies have reported similar *post hoc* findings[169,170,171].

Cohorts with metabolic syndrome or dysglycaemia

In the subjects with diabetes of the MrOS cohort[168], plasma (total) OC was negatively associated with total fat and trunk fat demonstrating that plasma OC is specifically associated with fat mass but not with lean mass. The negative association between OC and plasma glucose levels was strong while that between plasma OC and serum insulin was not as obvious in both all subjects and those without diabetes. Evaluation of serum lipids demonstrated that serum TG and HDL-cholesterol, but not total cholesterol or LDL-cholesterol, were moderately associated with plasma OC in all groups. In a smaller subgroup of subjects with diabetes (n=153) there was a similar trend of an association between OC and plasma glucose as seen for the larger subgroup of normoglycaemic subjects (n=857) but this trend did not reach statistical significance. No significant interaction between OC and diabetes (Yes/No) was observed for the association with variables relating to glucose homeostasis (FPG, HOMA index) or the different parameters reflecting body fat (total body fat, trunk fat and percent body fat, fat%). Thus, according to the authors, OC was clearly inversely associated with parameters reflecting fat mass and plasma glucose. According to this study, OC is an independent negative predictor of FPG and it is not mediated by insulin levels nor caused by T2DM or its treatments.

Additionally some smaller studies have reported similar findings and added novel observations for the effect of OC on lipids. *Zhou et al* who studied Chinese diabetes patients reported serum TG levels as an independent factor influencing OC in pre-menopausal women whereas age, fat%, HDL cholesterol, FPG and fasting serum insulin were independently correlated with OC in men[169].

Table 1 summarises the key clinical evidence indicating a relationship or association between OC (total, carboxylated or uncarboxylated) and markers of metabolic syndrome/disturbances, insulin resistance or other surrogate markers of energy metabolism. Most of the evidence comes from cross-sectional *post hoc* analyses of cohorts targeting other primary endpoints and thus these analyses preclude direct inferences concerning causality or a temporal relationship in a genuine longitudinal setting. Similarly, the indicated (mostly inverse) associations between total OC and the markers of metabolic impediments are modest, and the included cohorts recruited mostly individuals with subsequent confounding co-morbidities. In these analyses, the degree of OC carboxylation was seldom or reliably taken into account as uncarboxylated form of OC is not widely available or measured. Therefore, most of them incorrectly assume that total and uncarboxylated forms of OC have the same molecular-physiological role, thus introducing an unintentional bias in the literature used to support the model by *Karsenty et al*. The discrepancy among these studies may additionally be due to diversity within the evaluated clinical cohorts and the methods used to assess OC, but it could also be indicative of the age-dependency of the effect of OC on glucose metabolism among many other potential factors.

Table 1: Selected evidence demonstrating the inconsistency and heterogeneity of the clinical findings for confirmation or rejection of the proposed hypothesis of the role of osteocalcin on energy metabolism

Authors	Study population /methodology	OC type	Key findings
Pittas AG et al[167].	Post hoc analysis of healthy ambulatory 65+ year-old with osteoporosis, cross-sectional	Total OC only	FPG: inverse effect ↓ fS-INS: inverse effect↓ HOMA-IR: inverse effect ↓
Saleem U et al[172]	Post hoc analysis of a cross-sectional cohort with hypertensive adults belonging to same sibships, black and non-Hispanic whites with metabolic syndrome	Total OC only	FPG: inverse effect ↓ fS-INS: inverse effect↓ HOMA-IR: inverse effect ↓ Adiponectin: positive effect↑
Iki M et al[173]	Post hoc analysis of osteoporotic elderly 65+ year-old Japanese men	Intact and unOC	(unOC) FPG: inverse effect ↓ (unOC) fS-INS: inverse effect↓ (unOC) HOMA-IR: inverse effect ↓ (unOC) HbA1c: inverse effect ↓ Intact OC after adjustment for unOC: no effect on any parameters
Lu C et al[174]	Family-based three generation cohort (daughter- mother- maternal grandmother)	Total OC, cOC and unOC	HOMA-IR: no correlation for any OCs (total and cOC) Leptin: inverse effect ↓ in mothers (unOC) Leptin: inverse effect ↓ in grandmothers
Gower BA et al[175]	Prospective study of insulin sensitivity and beta cell response to glucose in obese adults	Total OC, unOC, %unOC	HOMA-IR: no effect/correlation for any OCs Reports a generalised relationship between multiple BTMs and bone; indicating a non-OC specific role of bone in T2DM

8. The aims of the research

Despite the growing body of evidence for the role of bone-derived OC in energy homeostasis there are yet no studies which would have evaluated a) how rapidly OC responses to changes in glucose homeostasis, b) whether these changes are biologically substantial and clinically relevant in normoglycaemic adults and c) how these changes associate with skeletal parameters.

Therefore, the specific aims of this research have been as follows:

1. to explore acute glucose-induced changes in bone metabolism and bone turnover markers in a cohort of young adults ***(I: 'Pilot')***

2. to explore the association between bone and glucose metabolism during a standard OGTT in healthy young adults born with VLBW vs. those born at term ***(II:'VLBW cohort')***

3. to compare multiple bone turnover markers between obese subjects with childhood-onset obesity and age- and sex-matched normal-weight controls and also, to evaluate the response of BTMs, including OC, to a rapid increase in circulating glucose levels, and the interplay between glucose, insulin and BTMs, during an OGTT ***(III: 'ELLU')***

4. to establish sex- and age-specific BTM reference intervals in healthy Finnish children and adolescents and to assess the variation by anthropometric variables, pubertal status and body composition on BTMs, especially on urinary, serum total and carboxylated OC levels ***(IV: 'normal reference range cohort')***

9. Materials and methods

9.1 Ethical considerations

All the studies were conducted in accordance with Declaration of Helsinki and the study protocols were approved by the Research Ethics Board of Helsinki University Central Hospital and/or Uusimaa Hospital District (HUS). The individual approval numbers are as follows: Study III (Dno 183/13/03/03/2010) and Study IV (HUS/212/E7//2005). Details for Study I and II can be retrieved from Petter Hovi et al.

All adult subjects provided a written informed consent (Studies I, II and III) while for all the paediatric subjects (in study III when applicable, and study IV) the consent was obtained from the parents and an assent from the children and adolescents. No additional invasive measurements beyond regular blood and urine sampling were imposed. The exposure to radiation during assessment of body and bone composition with dual X -ray was limited to the minimum. All subjects were informed about any clinically significant findings and referred to appropriate further medical care, if applicable.

9.2 Subjects and study designs

Study I was a pilot study (*'Pilot'*), which included the 23 sequentially first, more intensively studied, subjects enrolled in the VLBW cohort recruited through data from the Population Register Centre of Finland as explained below. This cohort, 12 females and 11 males, was pooled to represent a group of young adults close to the time of peak bone mass attainment to demonstrate the effect of acute glucose load on bone diverse turnover markers, independent of their past as infants with VLBW or their peers.

Study II/VLBW cohort consisted of 338 subjects, enrolled *by Hovi et al* to participate in an assessment of the various consequences of VLBW status later in life; premature VLBW subjects (n=166) and subjects born at term with normal birth weight (n=172)[122]. All subjects with available, consecutive serum samples at baseline and 2 hours during OGTT were included in the assessment of OC and metabolic parameters in this study.

- **Study I/Pilot and Study II/ VLBW cohort:** The original study cohort as collected *by Hovi et al*[122] comprised 335 consecutive, prematurely born (gestational age, GA < 37 weeks) VLBW infants born between January 1978 and December 1985. These subjects represented those discharged alive from the neonatal intensive care unit of Children's Hospital at Helsinki University Central Hospital, Finland. A comparison group was selected from the records of all consecutive births at each birth hospital among the following infants; the next available singleton born at term (GA ≥37 weeks) of the same sex and who was not small for GA (SD score for birth weight ≥−2.0). All the subjects were traced in 2004, in their young adulthood through data from the Population Register Centre of Finland. Mortality from hospital discharge to June 2004 was generally low, 1.8% for the VLBW subjects and 1.0% for the comparison group born at term. Birth weight ranged from 600 to 1500 g in the VLBW group and from 2560 to 4930 g in the term group; GA ranged from 24.0 to 35.6 weeks in the VLBW group and from 37.0 to 42.9 weeks in the term group. Among the survivors, 95.1% of VLBW subjects and 96.8% of subjects born at term were identified and a total of 255 VLBW subjects and 314 subjects born at term who were living in the greater Helsinki area were invited to participate in the study. A total of 338 subjects

agreed to participate; 166 of the VLBW subjects (65.1%) and 172 of the subjects born at term (54.8%). Subjects with T1DM (n=1), concomitant or chronic systemic glucocorticoid use (n=1), pregnancy (n=2) or insufficient fasting prior to OGTT (n=2) were excluded from the analyses.

Study III/ELLU (ELLU- Elintavat ja luusto nuorilla, i.e. in Finnish for *Lifestyle and skeleton in young subjects*) **cohort** assessed the skeletal and metabolic characteristics in 42 eligible subjects with severe childhood obesity, necessitating specialist care.

- Recruitment of subjects into ELLU started in 2011 and the main inclusion criteria for the enrolment of the targeted obese subjects included weight-for-height ratio exceeding 60% before 7 years of age (according to local, Finnish growth standards) and a referral due to severe childhood obesity to Children's Hospital, Helsinki University Central Hospital. The Subjects must have lived at the age of 7 years within the capital region of Helsinki and they were to be between 15 and 25 years of age at the time of inclusion in ELLU. In all, out of 230 eligible subjects who were invited to participate in the study, 42 (18%) subjects consented. All participating subjects had been followed by a paediatrician at the Children's Hospital and common endocrine and/or genetic causes of obesity had been excluded (e.g. Prader Willi syndrome, pseudohypoparathyroidism, hypercortisolism, hypothyroidism). Before first study visit two obese subjects withdrew their consent due lack of time, one due to pregnancy, and one due to diagnosis of T2DM. Additionally, OGTT was incomplete in three obese subjects. For each subject an age- and sex matched control was selected from the civil register, limited to the capital region of Helsinki. Exclusion criteria for the control subjects were obesity before age of 10 years (weight-for-height ratio above 40%). Altogether 35 controls provided a consent. However, in one subject OGTT was incomplete and thus a total of 34 age- and sex matched patient-control pairs, who had complete data, were included.

Study IV/ Normal reference range cohort was initiated in 2005 for cross-sectional, school-based assessment of the impact of exercise, nutrition and hereditary factors on bone health and/or related parameters in healthy children and adolescents (age between 7 and 19 years) in Helsinki region. For evaluation of serum OC, the final eligible cohort with sufficient sampling material consisted of 172 subjects.

- The original study comprised 195 children and adolescents who were recruited from randomly selected school classes in one primary and one secondary school in order to cover all age groups[176]. The aim was to reach a higher than a 60% participation rate. Participation in this study was voluntary and invitation letters were given by the teachers to the pupils and their parents. All those willing to participate were included. Our sub-analysis included a total of 172 subjects from the original cohort, 106 girls and 66 boys, who presented with normal bone mineral density (lumbar spine Z score between -2 and +2) and had retrievable data for clinical characteristics, including puberty stage, serum and urinary samples, and DXA parameters including whole body fat%. One outlier (a boy) was removed due to unreliable test result for an exceptionally high urinary OC value beyond reliable detection limit despite having met all the inclusion criteria. No additional exclusion criteria were introduced.

Table 2: Summary of the study cohorts and key analysis methods

Study cohort	Sample size (n)	Main inclusion criteria	Serum (S) / urinary (U) OC analysis	Other BTMs	OGTT	Bone / body composition analysis
I / Pilot	23	Healthy young adults	**x (S)**	**x[a]**	**x**	**x**
II / VLBW	337	Young adults born with VLBW or at term	**x(S)**	**-**	**x**	**x**
III / ELLU	34	Adolescents and young adults with childhood - obesity	**x (S)**	**x[a,(b**	**x**	**x**
IV / Normal reference range	172	Healthy children and adolescents	**x (S,U)**	**x[c**	**-**	**x**

a) Serum β-CTX, P1NP and TRACP5b,
b) BAP
c) Serum P1NP and serum ICTP, U-NTX, ALP

9.3 Methods

9.3.1 Assessment of clinical characteristics (I-IV)

All subjects in Study I-III were evaluated for anthropometry (weight, height, waist and hip circumferences, BMI). In Study I/II the subjects completed questionnaires mapping their past medical history, their regular leisure-time activity and exercise. In addition, details regarding the educational level of their parents or caregivers were recorded and the parent's history of diagnosed T1DM or T2DM. The blood pressure measurements were standardised and performed by measuring from the right arm of each subject. In Study III questionnaires were used to collect information regarding smoking, fractures and physical activity additional to recent history during the last 12 months focusing on commuting for school or work, guided or non-structured free time activity.

In Study IV targeting the paediatric population, information on medical and fracture history, medications, overall health, age at menarche, use of vitamin D and calcium supplements, and data about physical activity and dietary intakes were collected with a questionnaire. Height (cm), its SD and weight (kg) plus relative body weight (weight %) were measured and compared with Finnish growth charts[177]. No analysis

on ethnicity due to limited ethnic heterogeneity in this cohort was included on results presented for this research (as subjects were mostly Caucasians).

Based on serum gonadotropin and sex steroid concentrations, pubertal development was scored either as pre-, mid-, or post-pubertal by a paediatric endocrinologist. A united scale was introduced by converting the available Tanner stages into pre-, mid-, or post pubertal categories as follows: Tanner stages I–II were considered as pre-pubertal, stages III and IV mid-pubertal and stage V as post-pubertal.

The subjects were evaluated for clinical characteristics, medical history (including their parents' history of T1DM and T2DM) and for markers of adiposity and glucose metabolism.

9.3.2 Biochemistry

In all studies (I-IV) blood (serum /plasma) samples and second void urine were collected at 8–10 am after an overnight fast. Serum 25-hydroxyvitamin D (25-OHD) was assayed with high-performance liquid chromatography (HPLC, evaluated by Vitamin D External Quality Assessment Scheme, DEQAS), and plasma fasting parathyroid hormone (PTH) by an immunoluminometric method. 25-OHD, PTH and other biochemistry samples such as electrolytes, including (ion) calcium and potassium, were analysed as per validated standard protocols at HUSLAB.

Oral glucose tolerance test (I- III)

All subjects attended the clinic after an overnight fast of at least 10 hours. A 2-hour OGTT (75 g glucose) was initiated between 6:00 am and 11:15 am in Study I-III, depending on the schedule of the subjects. They ingested 75 g glucose solution, preferably within 5 minutes, after which plasma and serum samples were collected from a cannula inserted in an antecubital vein at baseline (0 min) and at 120 min in Study I and II, additionally at 30, 60 and 90 minutes in Study III.

Plasma glucose concentrations were measured by spectrophotometric hexokinase and glucose-6-phosphate dehydrogenase assay (Gluko-quant glucose/hexokinase, Roche Diagnostics) with a Hitachi Modular automatic analyzer at HUSLAB. At glucose concentration of 4.7 mmol per liter (84.7 mg per deciliter), the inter-assay coefficient of variation is 2.3%[178]. Impaired glucose tolerance was defined according to WHO as plasma glucose concentration ≥6.1 but <7.0 mmol/l at 0 min (fasting) and/or ≥7.8 mmol/l but <11.1 mmol/l at 120 min. Diabetes was defined by using standard criteria from international guidelines[179] as plasma glucose concentration ≥7.0 mmol/l at 0 min (fasting) and/or ≥11.1 mmol/l at 120 min. Serum samples were stored at -70 °C or colder until further analyses.

Based on OGTT findings, all subjects in study I and II were considered normoglycaemic at baseline (mean fasting plasma glucose ± SE 4.44 mmol/L ± 0.09 and mean fasting serum insulin 6.5 mU/l ± 0.60) and none had diabetes based on OGTT results. One subject in Study I with normal baseline glucose and insulin values had an increased glucose value at 120 min (9.2 mmol/l) and insulin value (288.0 mU/l) indicating signs of impaired glucose tolerance and driving the mean values for these parameters in the Study I cohort. At repeated testing both the 120 min both the glucose and insulin values were within normal.

In Study III population, 55% of the obese subjects and 3% of their controls had elevated fasting serum insulin concentrations (>12 mU/l) indicating increased insulin resistance or signs of pre-diabetic hyperinsulinemia. None of these subjects had a confirmed T2DM based on other glycaemic parameters.

Adiponectin (II-III) and leptin (III)

Adiponectin was assessed as an exploratory parameter for the subjects in Study II (unpublished data) and Study III. Serum adiponectin was determined with Human Total Adiponectin/Acrp30 Quantikine ELISA Kit and serum leptin with Human Leptin R Quantikine ELISA Kit (R&D Systems, Minneapolis, USA) with intra- and inter- assay CV of < 12%.

Measurement of insulin, HOMA-IR and HbA1c (II, III)

Serum insulin was measured with time-resolved immunofluorometric assay (Perkin Elmer Life Sciences, Finland) with a detection limit of 0.5 mU per liter (3 pmol per liter) and an inter-assay coefficient of variation of less than 4%[180]. The insulin-resistance index determined by homeostasis model assessment (HOMA-IR) was calculated as the product of the fasting serum insulin concentration (in milliunits per litre) and fasting plasma glucose concentration (in millimoles per liter) divided by 22.5[181]. The glycosylated haemoglobin (HbA1c) was measured by direct photometric immunoassay from serum (in %, normal range 4-6%) at HUSLAB according to standard protocols.

9.3.3 Bone turnover markers (I-IV)

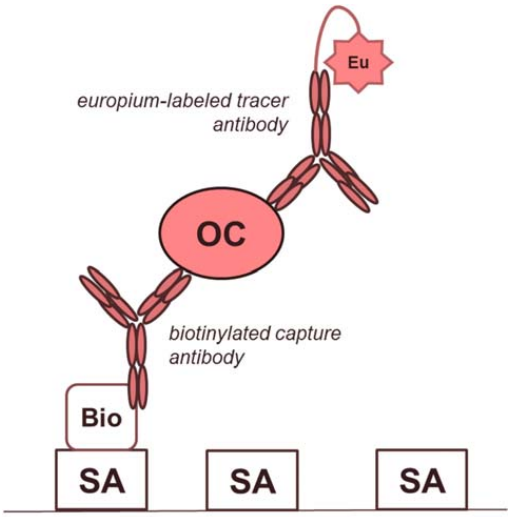
Serum osteocalcin (I-IV)

Serum total OC and cOC were determined at 0 min and 120 min in an OGTT by previously described two-site immunoassay protocols with monoclonal antibodies (Mab)[182]. Two Mab-Mab combinations were selected on the basis of previous, validated assays and modified by the undersigned based on test calibration runs in order to reduce the background signal. The two-site immunoassay for serum total OC is based on Mabs 2H9 and 6F9 and detects the large N-terminal mid-segment of the OC molecule. Assay for cOC utilised the Mabs 6F9 and 3H8, which detect the same fragments but prefer γ-carboxyglutamic acid (Gla) containing forms of OC, with <10% cross-reactivity to completely undercarboxylated OC (i.e. unOC)[183]. The Mabs were raised either against bovine OC (3H8) or a fusion protein consisting of glutathione-S-transferase or human OC (6F9 and 2H9). The Mabs were prepared and provided for these analyses by the University of Turku, Institute of Biomedicine.

In the assay, 200 ng of biotinylated capture Mab and 100 ng of europium-labelled tracer Mab per well were used. Synthetic peptide of human OC amino acids 1-49 (Advanced Chemtech, USA) was used as a calibrator. Streptavidin-coated microtiter plates were from Kaivogen (Turku, Finland) and other immunoassay reagents (Delfia® Assay Buffer, Wash Solution and Enhacement solution) from Perkin Elmer Life Sciences (Turku, Finland). Time-resolved fluorescence was measured with Victor2 Multilabel Counter (PerkinElmer Life Sciences). All samples were measured as duplicates and simultaneously at the end of each study. In order to reduce the bias by inter-assay variability, samples for each subject (0 min and 120 min) were analysed in parallel. The intra- and inter-assay variations were 4.4% and 9.0%, for total OC and 2.5% and 8.0% for cOC, respectively.

In study II with VLBW cohort, due to lack of validated method for accurate analysis of unOC values, we used a calculated value for uncarboxylated OC, which was derived from total OC and cOC values (unOC = total OC minus cOC).

Figure 5: The principle of two-site osteocalcin immunoassays



SA; Streptavidin –coated wells on a microtiter plate, Bio; biotinylated Mab, OC; osteocalcin from sample or standard, Eu; europium-labeled Mab

Urinary osteocalcin (IV)

Urinary osteocalcin was determined with a two-site assay for osteocalcin mid-fragment (U-MidOC), which is based on the same Mab –binding principle as the serum assays for OC. The samples were collected as the first morning void and the results were normalized for urinary creatinine determined with the alkaline picrate reaction and expressed as ratios. The calibration series covered the range from 0.3 to 90 µg/L and any samples beyond the upper limit were considered unreliable. The within-assay (CVa) and between-assay (CVi) variations for the assays were 1.7 and <12% for U-MidOC. All analyses were performed blinded and in duplicates. The samples for each time point were analysed simultaneously to minimise inter-assay variability.

Other bone turnover markers (I, III, IV)

In Study I and III bone resorption was assessed by measuring serum levels of βCTX-I and TRACP5b using commercial, automated Serum CrossLaps® ELISA and BoneTRAP® Assay, respectively (both using IDS Ltd, UK). Bone formation was assessed by measuring serum intact P1NP using automated analyser IDS-iSYS Intact P1NP assay (IDS Ltd, UK). Samples were analysed blinded and as duplicates, except for P1NP which was analysed using single sample due to insufficient sample volume for duplicate testing. According to the manufacturer, intra- and inter-assay variations for the assays are for βCTX-I <6% and <10%, for TRACP5b <6% and <6% and for P1NP <4% and <6%, respectively. The BTM

measurements in Study I were performed by Pharmatest (Oulu, Finland) and in Study III by ValiRx Finland Ltd (Oulu, Finland).

In Study IV Serum intact N-terminal propeptides of type I collagen (S-P1NP), Serum Type I collagen carboxyterminal telopeptide (S-ICTP) and alkaline phosphatase (ALP) were determined per validated standard protocols of the Central Laboratory of Helsinki University Central Hospital as described elsewhere [184]. Concentration of type I collagen crosslinked N-telopeptide (INTP or NTx) was measured from second void urine samples with Osteomark® NTx Urine Enzyme-linked Immunosorbent Assay (Alere Scarborough, Inc., Scarborough, USA) with intra- and inter-assay CV% < 8. The given values (nM Bone Collagen Equivalents) were corrected with urinary creatinine concentration (nM).

9.3.4 Bone and body composition analysis (II, III, IV)

In Study II, bone mineral content (BMC) and areal BMD (aBMD) for the lumbar spine (L1–L4) were measured with dual-energy X-ray absorptiometry (DXA, Hologic Discovery A, software version 12.3:3) and transformed into age-adjusted Z scores using the equipment-, age-, and sex-specific reference data. A cut-off Z score value of -1.0 was chosen to define reduced BMD. Body composition, including lean body mass and fat percent (fat-%), was determined with the same DXA equipment.

A total of 284 subjects (of 332) were available for the analysis of lumbar spine aBMD, which was chosen as the site for analysis due to its high content of trabecular bone and active turnover. The reasons for the missing BMD results included unwillingness to undergo DXA, pregnancy, cerebral palsy, several compressed lumbar vertebrae or foreign objects in the scanning area. Scans with foreign objects such as surgical fixation material or jewellery in the measurement area (five subjects) were omitted from the analysis. If more than one lumbar vertebra was compressed, the corresponding lumbar spine scan was excluded from the analysis (two subjects); if only one lumbar vertebra was compressed, BMD without the affected vertebra was used (seven subjects).

In Study III, whole body bone area (WB BA) was measured with Lunar Prodigy Advance DXA. Calibration was performed with a spine phantom; inter-CV% for BA was 0.38%. Reducibility of DXA measurement for total body is: BMD= 0.63%, BMC= 0.45% and BA= 0.78%[185]. Four obese subjects exceeded the maximum weight for the DXA device (160 kg) and therefore the mean WB BA value for all obese subjects (2570 cm^2) was used for them in the multivariate data analyses.

In Study IV, BMD, BMC and WB BA, were measured with DXA; Hologic Discovery A, paediatric software, version 12.4, from the LS (L1-L4), total hip and WB. DXA measurements were performed within three months of the biochemical sampling. All measured values were transformed into Z-scores using the equipment-specific age- and sex-adjusted reference data for US Caucasian children; all subjects were of normal height[186]. Body composition was analysed to obtain lean body and fat mass. Calibration of the measurements was performed by using a spine phantom; inter-CV% for the phantom BMC, area, and BMD were 0.35%, 0.21%, and 0.41%, respectively. The reproducibility of the DXA measurement for bone, fat, and lean mass is 1.2%, 1.9% and 0.7%, respectively, in children between 10 and 18 years of age[187].

9.3.5 Statistics (I-IV)

The Shapiro–Wilk test was used to test for normality, and nonparametric tests were used to determine associations between BTMs as they were not normally distributed (Shapiro–Wilk test<0.95). Glucose, insulin, adiponectin and all OC concentrations, and HOMA-IR were non-normally distributed and were analysed after logarithmic transformation.

Most parameters for descriptive statistics are presented as geometrical mean values ± SD or SEM, when appropriate. Z scores for skeletal parameters are indicated especially in paediatric cohorts. A paired t test was used to detect longitudinal changes during OGTT in each variable. Spearman's correlation was used to study the associations between OGTT-induced changes (Study I-III). Linear regression was used to study associations between different BTMs and between other parameters and/or sampling time-points.

The difference between parameters for the Term and VLBW groups (Study II) were analysed with one-way ANOVA. Standardised linear regression coefficients (β_{std}) between the OGTT analytes or the 120 min changes in the analytes were determined using linear regression. Multivariable regression analysis was used in all studies, adjusting for clinical characteristics such as age, gender, VLBW status or BMD.

We used SPSS for Windows; versions between 16.0-21.0 (SPSS Inc., Chicago, IL) for statistical analyses, except for linear regression which were calculated using Statistica for Windows 7.1 (StatSoft Inc., Tulsa, OK). P values <0.05 were considered statistically significant.

Also, the previously unpublished adiponectin data were used for simple linear regression and multiple variable analyses only after natural logarithmic transformation (concentrations non-normally distributed).

10. Results

10.1 Clinical characteristics of all study participants (I-IV)

The clinical demographic characteristics of all study participants are presented in Table 3. The overall, combined cohort (n=572) represents a diverse but singular population, in which the role of OC has now been characterised both as 1) a normative BTM reflecting the diverse stages of normal growth spurt through puberty and early adulthood, and 2) a potential key player in glucose homeostasis in apparently healthy individuals, but also in those with a history of childhood metabolic conditions such as morbid obesity.

Subjects in Study IV were the youngest, with mean age of 13.8 years (for girls) and 12.5 years (for boys), representing conveniently the time around the achievement of mid-puberty status: mean age for mid-pubertal groups was 12.8±1.27 years and 13.8±1.96 years for girls and boys, respectively. The mean age category enrolled represented also the most heterogeneous group of different stages of puberty (pre-, mid- and post- as displayed for our cohort in Figure 6), especially in boys. In this paediatric cohort with such large age-related disparity it was considered pragmatic to divide the cohort in more robust age categories for both sexes when defining age-specific reference ranges. The graphical presentation (Figure 7) illustrates how the wide age range for girls (7A) can be divided into five balanced age categories (7B), in which the age-dependent increase in height (7C) or weight (7E) demonstrates an equal distribution of height SD (7D) or weight % (7F) across over all age categories.

All cohorts enrolled represented essentially normoglycaemic individuals with no previously diagnosed impairment of glucose tolerance. In Study I, one subject with a normal baseline glucose value had an increased glucose value at 120 min (9.2 mmol/l) indicating signs of IGT but after repeated testing the 120 min value was within normal limits. Many obese subjects in Study III presented with early signs of insulin resistance (as discussed later in 10.5.3). In the same study, even if the mean plasma glucose concentrations were similar between the groups at any assessed time point (AUC in repeated measures ANOVA; P=0.066), 3-fold higher serum insulin concentrations were observed in response to OGTT in obese subjects compared with controls (AUC in repeated measures ANOVA; P =0.003). At 120 min glucose values were within normoglycaemic range (< 7.8 mmol/l) in all subjects.

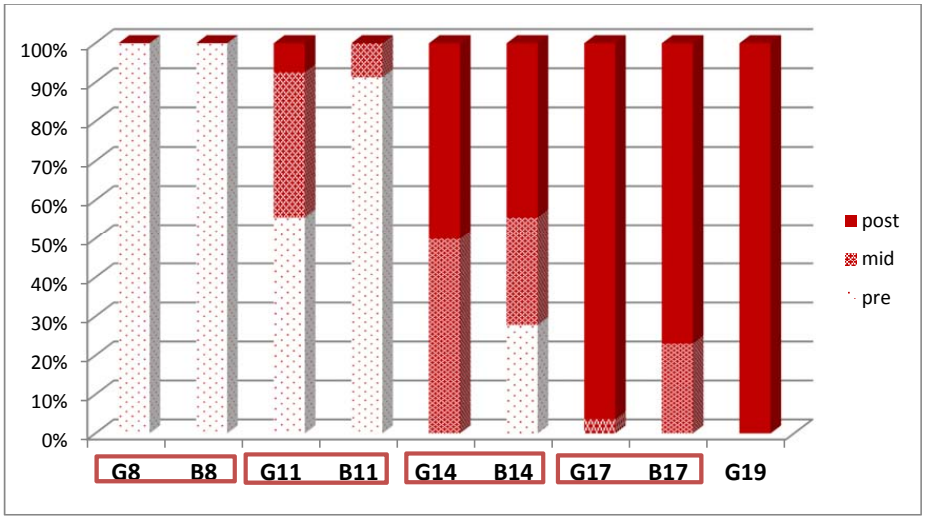
In general, even if normoglycaemic, subjects with VLBW had higher 2-hour insulin (p=0.001) and 2-hour glucose concentrations (p=0.037) and marginally elevated fasting insulin (p=0.068) as compared with those born at term.

Table 3: Clinical characteristics of the study populations (n=572)

	Study IV		Study III		Study I/II	
Cohort/n	**Girls** (n=106)	**Boys** (n=66)	**Obese** (n=34)	**Controls** (n=34)	**VLBW** (n=163)	**Controls** (n=169)
Age (years)	13.8±2.7	12.5±2.7	19.3 ± 2.3	19.4±2.3	22.4 ± 2.1	22.5 ± 2.2
Weight (kg)	48.0 ± 12.6	48.4 ± 18.2	120.9 (27.7)	64.8 (12.1)	67.2 ± 13.1 (M) 58.5 ± 12.0 (F)	76.1 ± 67.2 (M) 63.5 ± 10.8 (F)
Weight%	5.6 ± 16.4	8.5 ± 18.5	-	-	-	-
Height (cm)	157.3 ± 11.8	154.9 ± 18.0	173.3 (8.6)		174.6 ± 7.7 (M) 162.0 ± 7.6 (F)	180.5 ± 6.4 (M) 167.2 ± 6.8 (F)
Height SD	0.32 ± 0.94	0.50 ± 1.07			-	-
BMI (kg/m^2)	19.1 ± 3.28	19.5 ± 3.83	40.4 (9.4)	21.9 (3.1)	22.0 ± 3.6 (M) 22.3 ± 3.9 (F)	23.3 ± 3.2 (M) 22.7 (3.7) (F)
Glycaemia						
Status	NGT	NGT	NGT*	NGT	NGT	NGT
FPG (mmol/l)	-	-	5.3 ± 0.43	5.1 ± 0.56	4.7 ± 1.1	4.7 ± 1.1
HbA1c (%)	-	-	5.30 (0.31)	5.17 (0.23)	-	-
Insulin (mU/l)	-	-	15.69 (9.43)	6.31 (3.05)	5.6 (1.7)	5.1 (1.7)
BTMs (ng/ml)						
Total OC	26.6 ± 14.0	30.8 ± 10.2	13.61 (5.21)	17.85 (9.62)	12.4 (1.5)	11.3 (1.5)
cOC	32.8 ± 15.3	39.2 ± 12.3	13.14 (5.07)	17.49 (9.36)	12.0 (1.5)	10.6 (1.5)
U-OC	11.6 ± 7.66	16.0 ± 7.4	-	-	-	-
B-CTX	-	-	0.73 (0.36)	1.02 ± 0.64	0.75±0.33	(pooled: Study I)
P1NP	422.6 ± 327.6	544.7 ± 231.2	82.0 (43.1)	121.4 (110.8)	66.0±46.1	(pooled: Study I)
BMD						
WB Z score	0.18 ± 0.60	0.30 ± 0.61	-	-	-	-
WB BA (m^3)			2573 (318)	2303 (287)	-	-
LS areal Z score			-	-	-0.94 (0.98)	-0.42 (1.05)
Males (%)	0	100	41	41	44	40

*Parameters are presented as mean values ±SD when applicable. *indicates potential inclusion of single metabolically pre-diabetic subjects, (-) indicates non-available or not applicable parameter, (M) for males and (F) for females, if assessed separately*

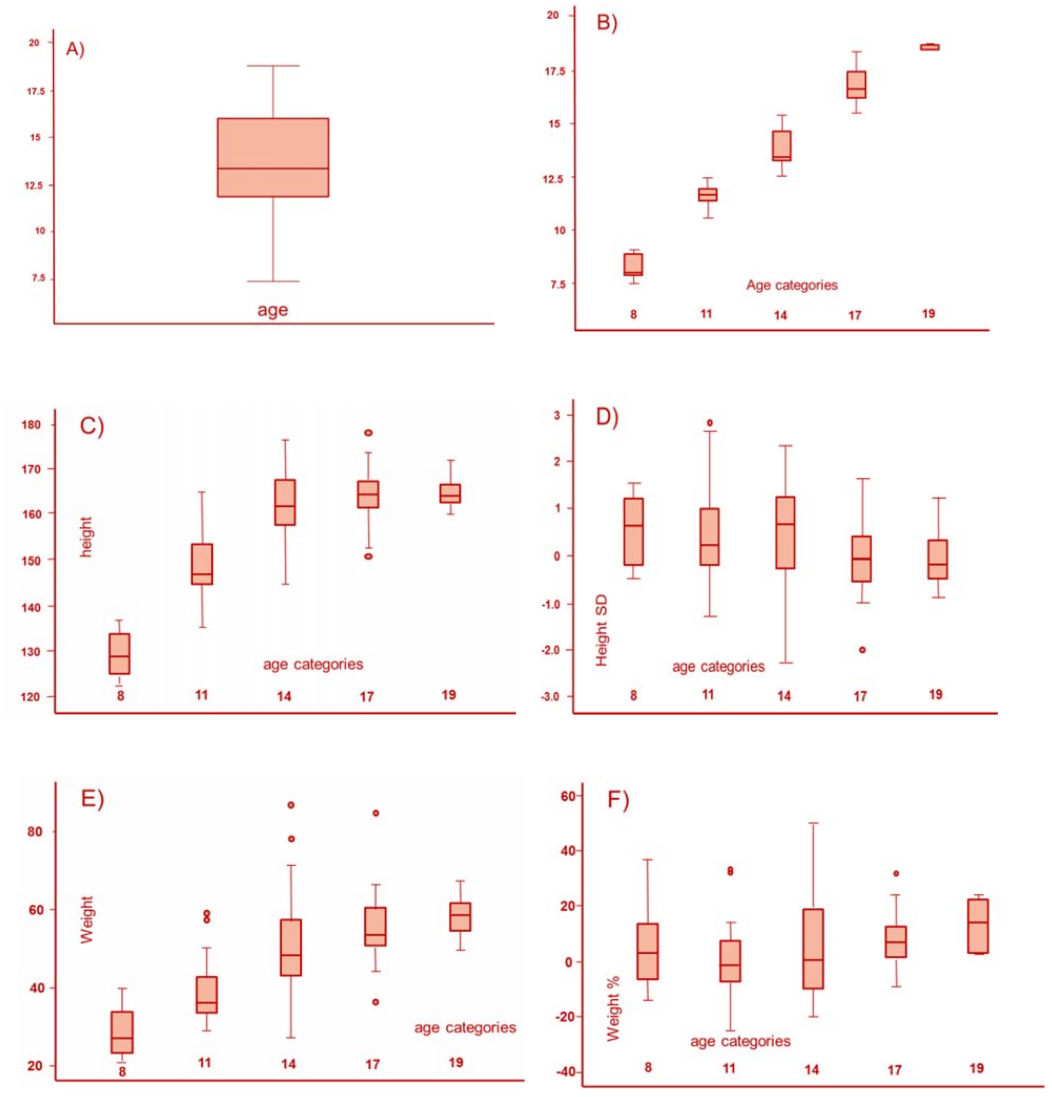
Figure 6: Proportional pubertal stage (pre-, mid-, post-) status for girls (G) and boys (B) per age category.



In Study IV, the mean serum 25-OHD (±SD) concentration, 42.5±12.46 nmol/l (range 17.0 nmol/l, 82.0 nmol/l for all pooled girls and boys, n=171), was considered low, even insufficient, while apparently also reflecting the time-point of assessment, the winter period. All study populations were also assessed for skeletal parameters, both BTMs and DXA-based estimates of specific skeletal sites such as LS or FN for Study II or WB Z score and/or WB BA, which are more appropriate for paediatric populations as those enrolled in Study III or IV. For Study IV, individuals with WB Z score beyond +2 or -2 were excluded. The mean results of the DXA analysis in addition to the anthropometric clinical characteristics confirmed that these study populations represented mostly apparently healthy individuals, children, adolescents and young adults, with normal bone metabolism.

Presenting mean values of anthropometric or skeletal parameters for a heterogeneous, healthy and mostly growing paediatric cohort ranging from 7 to 18 years of age, as for Study IV and in Table 3, is only justified by the purpose of this general composite presentation of the overall demographics for the subjects enrolled for this research.

Figure 7: Distribution of clinical characteristics (age; A/B, height and SD; C/D, weight and wt%;E/ F) for girls in Study IV per age category



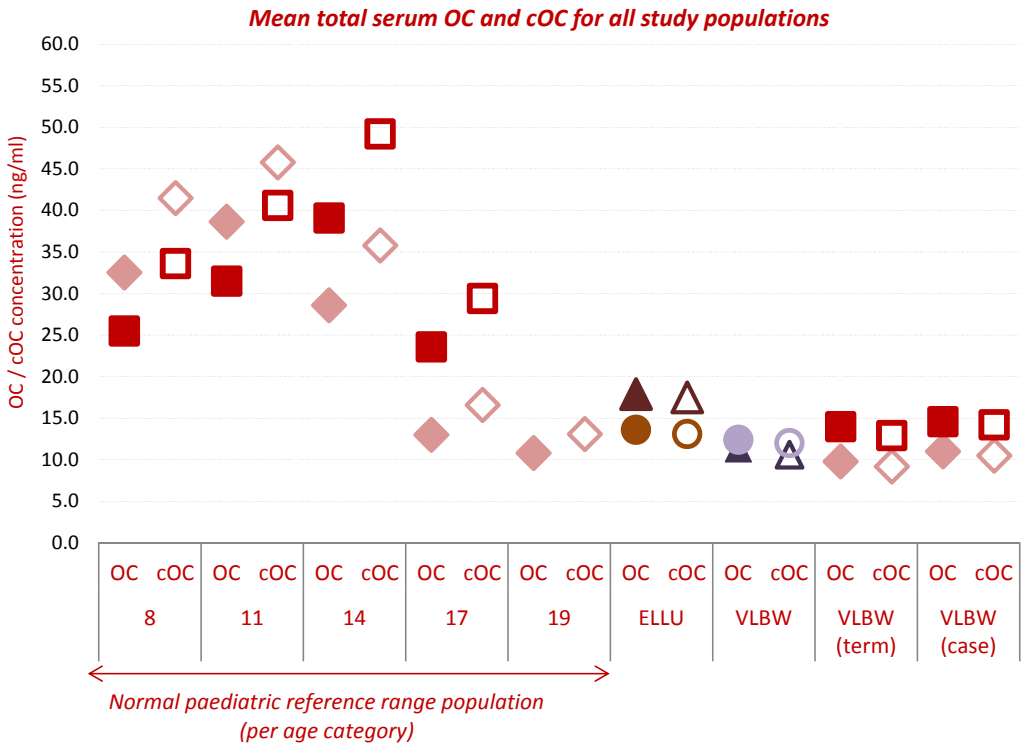
10.2 Serum osteocalcin levels in children, adolescents and young adults (I-IV)

The mean fasting OC and cOC concentrations are presented for all study cohorts in Table 3 and Figure 8. In childhood, the OC and cOC values for both girls and boys reflect the expected BTM patterns for a normal pre-pubertal growth phase and new bone formation through modeling; the mean OC values were between 25-35 ng/ml, with mean cOC values incrementally, up to 10 ng/ml, higher. During the peak growth spurt for girls in age category of 11, the OC and cOC concentrations are elevated up to 350% vs. the corresponding concentrations during post-pubertal phase (age categories 17 and 19 in girls) or later in life as measured in the young adult cohorts. The normal OC and cOC concentrations for adult female subjects beyond age of 20 years stabilised around 9-10 ng/ml and 13-14 ng/ml, respectively. As reported for Study II, the fasting OC and cOC concentrations were statistically significantly affected by the VLBW status ($p=0.027$ and $p=0.005$ for OC and cOC, respectively) even if these differences might not be clinically relevant.

The same pattern of OC dynamics was observed for boys and male subjects while the timing of the growth spurt and peak concentrations of OC and cOC in boys appear later than in girls, in the age category of 14 years. The cOC concentrations are raised up to 380% vs. those values determined as normal for young adult males. The elevated mean OC concentrations (mean 23.6 ng/ml) and also the highest SD in all OC categories (10.43) in age category 17 reflect the number of still mid-pubertal and growing boys in the same group.

Mean age (19.3/19.4 years) in the ELLU cohort corresponds with the age category of 19 years for girls in Study IV while the overall OC and cOC concentrations in the ELLU cohort, both for those with a history of childhood obesity (total OC: 13.6±5.21 ng/ml) and for their controls (total OC: 17.6±9.62 ng/ml), seem slightly higher vs. the age category 19 or the Study I/II cohorts. This type of variation confirms how sensitive the BTM assays are and how even the slightest deviation or difference in the analysis methodology, or other environmental factor as discussed previously, may alter the results of the apparently same analysis on a different day or cohort.

Figure 8: Overall presentation of pooled mean serum OC (solid marker fill) and cOC (no marker fill) concentrations for all study cohorts: paediatric normal reference range cohort per age categories (8-19: Study IV, females subjects with pink diamonds and males subjects with red rectangles), ELLU cohort (Study III) for obese (brown circles) and control (brown triangles) subjects and Study I for those born at term (violet triangles) or with VLBW (lilac circles) or indicated separately for Study II cohort: females subjects with pink diamonds and males subjects with red rectangles.



10.3 Effect of acute glucose load during OGTT (I- III)

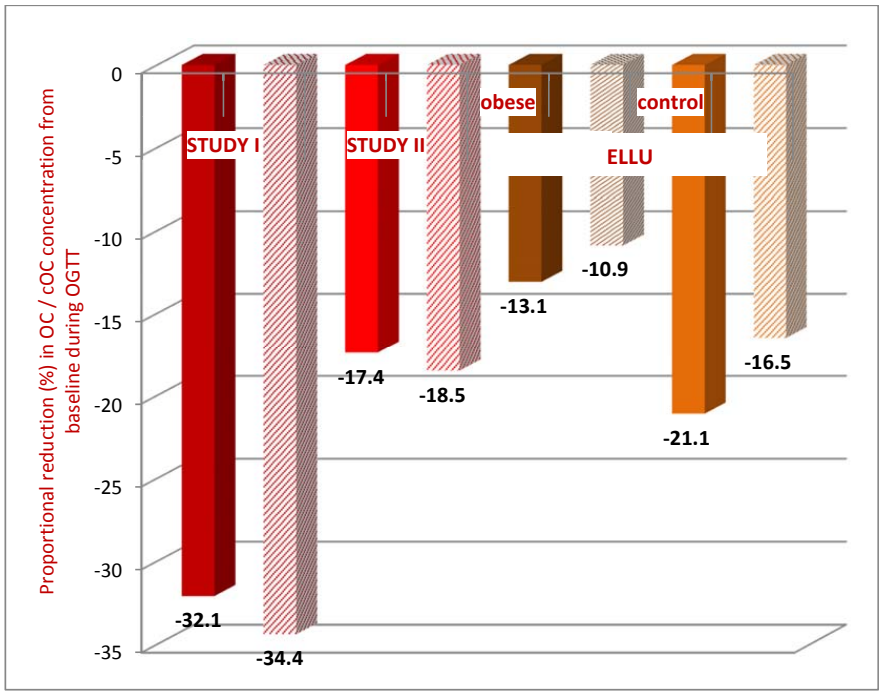
10.3.1 Impact of OGTT on serum total OC and cOC

The mean baseline concentrations of total OC and cOC are displayed for all study cohorts in Table 3. As analysed in detail for Study I, the observed OC levels were independent of the time of collection: standardised regression coefficients for OC levels and sampling time (between 8:05am and 11:02am) were for OC 0.33 (p=0.128) and for cOC 0.40 (p=0.06).

Overall, the OGTT induced a significant decrease in both OC and cOC concentrations for all study cohort groups (Figure 9). There was a strong association between the changes in total OC and cOC (Study I: Spearman's correlation, r=0.83, p< 0.001) while the magnitude of the reduction from baseline varied between cohorts (Study I vs. Study II-III) and within cohorts (Study III obese vs. controls). Unexpectedly, the most pronounced decline in OC and cOC concentrations were induced in the Study I pilot population evaluated: the median (interquartile range; IQR) decreases for OC and cOC were -32.1% (-37.9 – -19.6) and -34.4% (-39.8 – -22.2), respectively. These samples were collected from the firstly enrolled 23 subjects, otherwise representative of the overall pooled Study I-II population and thus not providing no physiological reasons for the more pronounced reduction in OC/cOC levels vs. the overall cohort.

However, the dynamics of total OC and cOC during OGTT demonstrated an unexpected pattern between the groups in Study III when the maximum decreased in obese subjects was limited to -13.1% and -10.9% for total OC and cOC, respectively, while the equivalent reductions in the control group were significantly higher, i.e. -21.1% (P=0.022) and -16.5% (p=0.055), respectively. Nevertheless, due to the already lower baseline OC levels (Table 3) the relative decrease in total OC during OGTT was less distinct in obese subjects vs. controls (p=0.029). There was no difference between groups regarding cOC concentrations (p=0.139). In addition, even if the postprandial total OC was suppressed, the effect appeared later in obese subjects than in controls.

Figure 9: The OGTT-induced effect on serum total OC (solid colour) and cOC (striped). Assessment for study I (dark red) and Study II (red) cohorts at 120' and Study III (dark brown for obese and lighter brown for controls) at the time of maximum postprandial effect (60'-120' depending on the variable)



10.3.2 Effect of OGTT on other BTMs (I, III)

In Study I, the only post-ingestion time-point gauged for BTMs was 120' while in Study III the OGTT also included 30', 60' and 90' time-points. Figure 10 summarises the effect of OGTT on reference BTMs in Study I and III.

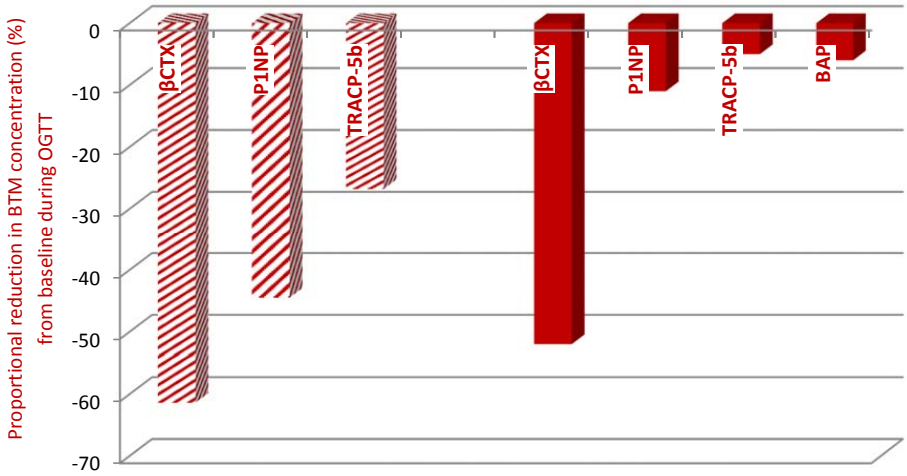
For Study I, the OGTT resulted in a statistically significant decrease from baseline to 120' in all evaluated BTMs (paired t-test, $p < 0.001$ for all variables). The reductions (IQR) for the resorption markers βCTX-I and TRACP5b were 61.4% (68.5, 53.0) and 44.5% (48.3, 40.2), respectively, while the bone formation marker PINP was reduced with -26.8% (33.2, 19.2). In Study III the most consistent reductions were being observed for CTX additional to OC, with maximum reduction of 52% for CTX.

As expected, all BTMs in Study III responded rapidly within 30 minutes and demonstrated maximum drops from baseline between 60 and 90 minutes ($p<0.02$ for all with no difference between the studied groups [p-values between 0.5 and 0.7 for the comparison]). The post-OGTT changes in Study I for βCTX-I and TRACP5b were not associated with changes in serum OC or serum cOC ($r=0.22$, $p=0.31$ and $r=0.26$,

p=0.24, respectively) while the change in βCTX-I was moderately associated with reduction in PINP (r=0.42, p=0.047) and TRACP5b levels (r=0.44, p=0.037).

None of the OGTT-induced changes in BTMs were associated with OGTT-induced changes in insulin or glucose alone in either Study I or in Study III (p>0.05 for all). However, inverse associations between acute (r= -0.315, p=0.020) and steady-state (r= -0.380, p=0.007) responses of BAP to glucose were observed in Study III; the association remained significant after adjusting for insulin (r= -0.301, p=0.027). Correspondingly, acute change in glucose was inversely related to overall response (measured as AUC) in CTX but only with a borderline level of significance (r=-0.208, p=0.059).

Figure 10: Impact of acute glucose load (standard OGTT) on different BMTs in Study I at 120' (striped) and Study III (maximum observed drop: solid colour).



10.3.3 Osteocalcin, glucose and insulin (II)

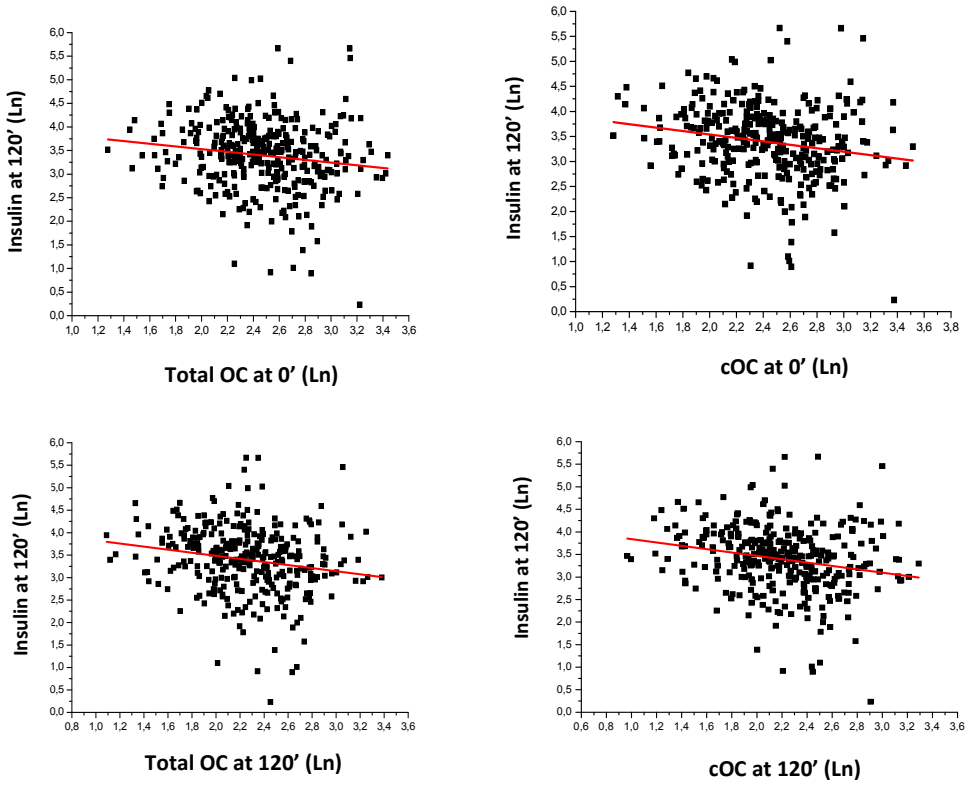
In Study II, fasting glucose and insulin concentrations were within normal ranges in both VLBW and control groups while insulin levels were marginally higher in VLBW subjects (+11%, p=0.068) when compared to those born at term. This difference between groups was also statistically significant between the female VLBW subjects and their controls; females with VLBW had higher baseline insulin values (5.8 vs. 5.0 mU/L, p=0.045). There was no difference in fasting glucose levels between the groups (p=0.24) or between the genders. Yet, at 120' all subjects with VLBW had significantly higher unadjusted insulin (+30.5%, p=0.001) and glucose concentrations (+5.5%, p=0.037) than those born at term as also previously reported by *Hovi et al*[122].

In the overall cohort, fasting total OC and cOC were negatively correlated with fasting insulin levels after adjustment for age, gender, VLBW status and BMD (r= -0.182, p=0.009 and r= -0.283, p<0.001, respectively). The observed correlation persisted as significant even after adjustment for lean body mass (r= -0.242, p<0.001) and WB fat% (r= -0.144, p=0.028). The correlation between fasting insulin and OC was similar in both groups, although the association parameters were generally stronger in the VLBW group. There was no significant association between insulin and total OC when the correlations were further adjusted for body fat%.

During OGTT, the correlation between the changes in glucose and insulin concentrations at 120' were as expected significant (r= 0.663, 95% CI 0.582, 0.745, p<0.001). Total OC and cOC were only modestly associated with postprandial levels of glucose (p=0.022 and p=0.034, respectively) but not with insulin (p=0.54 or 0.24, respectively). Figure 11 displays graphically the lack of correlations between these key parameters, total OC and cOC (at 0' and 120') and postprandial insulin levels.

The lack of existing, validated methods for the accurate analysis of the proportion of uncarboxylated OC, unOC, has limited investigation of the unOC values derived from theoretical estimates based on the total OC and cOC measurements. In our analysis set, fasting insulin levels correlated positively with calculated proportion of unOC (r=0.176, p=0.001) and this correlation was significant also after adjustment for age, gender and VLBW status (r=0.193, p<0.001). Against the proposed theory, the total OC correlated negatively and the estimated unOC positively with fasting insulin when adjusted for age, gender, VLBW status and lean body mass (r=-0.156, p=0.021 and r=0.153, p=0.007, respectively).

Figure 11: Correlations (unadjusted) between 0’ or 120’ serum total OC and cOC, and postprandial 120’ insulin concentrations. The lack of association between postprandial insulin levels and serum OC concentrations (total or cOC, at 0’ or 120’) indicates that insulin is not a key mediator of acute glucose homeostasis (all p=ns). For details of correlation parameters, see text.

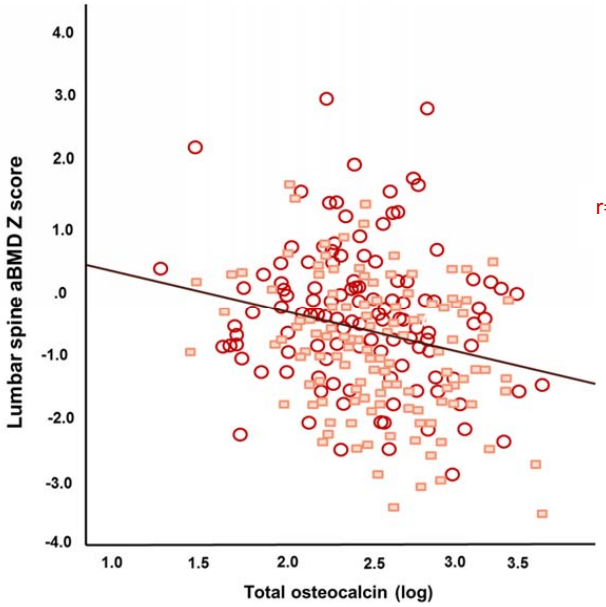


10.4 Osteocalcin and BMD (II)

In the Study II population, the mean areal LS Z-score values were significantly lower in the VLBW subjects than in those born at term (-0.94± 0.98 vs. -0.42 ±1.05, respectively, p<0.001). Similarly, the aBMD Z score for FN was reduced in the VLBW cohort (-0.48 ± 0.86) while it was normal in those born at term (0.14 ± 0.98, difference p<0.001). Consistently, the fasting OC and cOC concentrations were also significantly higher in subjects born with VLBW vs. their controls (ANOVA, p=0.027 and p=0.005, respectively). Independently, both groups showed a similar and statistically significant correlation between the total OC and LS areal BMD (pooled): r=-239, p<0.001 (Figure 12).

In a multiple regression model for paediatric population (Study IV) the WB BMD Z score was not an independent predictor of serum total OC (p=0.404) or cOC (p=0.932) concentrations.

Figure 12: Association between total OC and LS aBMD Z -score in overall population with pooled young adults (Study II): controls (dark red circles) and subjects with VLBW (pink rectangular markers).



10.5 OC and markers of metabolic or glycaemic disturbances (I-III)

10.5.1 HOMA and serum insulin (II-III)

Fasting serum insulin is a driver of homeostasis model assessment of insulin resistance index HOMA-IR[181]. In Study II cohort, the assessed HOMA-IR values were normal: 1.0 ± 1.75 and 1.12 ± 1.85 for females and males, respectively. The difference between HOMA values in VLBW (1.37 ± 0.86) and control (1.27 ± 1.02) groups in fasting state did not reach statistical significance (ANOVA, p=0.342). HOMA-IR strongly correlated with fasting OC and cOC, independent of age, gender, VLBW status and height ($p<0.001$ for all correlations) and independent of lumbar spine BMD ($r=0.180$, $p=0.010$ and $r=-0.277$, $p<0.001$ for total OC and cOC, respectively). The association between HOMA-IR and total OC was no longer significant ($p>0.05$) when further adjusted for BMI, whereas association with cOC remained significant ($r=-0.126$, $p=0.038$).

In Study III the baseline HOMA values indicated elevated insulin resistance in obese subjects and was significantly higher in obese (3.89 ± 2.36) vs. controls (1.46 ± 0.77), $p<0.001$.

10.5.2 Adiponectin (II-III) and leptin (III)

The mean adiponectin concentrations for the Study II (unpublished data) and Study III cohorts are presented in Table 4. The levels of adiponectin were similar for both VLBW and control groups, falling in between the mean values for obese and control subjects in Study III. There was a 3.6%, non-significant, difference in the adiponectin concentrations between the control and VLBW cohorts in favour of the VLBW cohort. The adiponectin concentrations were significantly higher in the control subjects of Study III vs. obese ($p=0.004$).

Table 4: Adiponectin and leptin concentrations in Study II and III

cohort	STUDY II		STUDY III	
	VLBW	*Control*	*Obese*	*Control*
Adiponectin (SD), *ng/ml*	8116 (1577)	7833 (1641)	7734 (3115)	11625 (6499)
Leptin (SD), *pg/ml*	-		51887 (28990)	8875 (6995)

In Study III, total OC and cOC were inversely associated with adiponectin concentrations at baseline (Spearman correlation -0.418, $p=0.017$ and -0.452, $p=0.011$ for OC and cOC, respectively). In Study II a simple, unadjusted linear regression model demonstrated no significant association between adiponectin concentrations and serum levels of the tested candidate influencing the energy metabolism, cOC ($r= -0.055$, $p=0.321$). In a multiple regression analysis when adjusting for clinical characteristics such as age, sex, anthropometric or parameters reflecting the body composition or skeletal health, there

was a trend (p=0.072) towards an association between cOC and adiponectin in all subjects. Otherwise, as expected, adiponectin was associated with age, sex and BMI (all p<0.0001) while also LS aBMD Z score (p=0.008) seemed to correlate with adiponectin concentrations.

Adiponectin was not related to any BTMs in the obese cohort (all p=ns) in Study III, but in the control group its concentrations were inversely related to all measured BTMs (total OC, cOC, BAP, PINP, TRACP-5b; all significant), except CTX (p=0.071). In the combined cohort, adiponectin was only related to BAP (Spearman correlation -0.399, p<0.001) and tended to relate with PINP (-0.249, p=0.051). In both groups and in the combined cohort, adiponectin was inversely associated with insulin (-0.568, p<0.001) and glucose (-0.373, p=0.002).

Leptin concentrations in Study III inversely associated with those of adiponectin (-0.304, p=0.012) in the overall pooled population but not for obese or controls alone. It was significantly inversely correlated with all of the assessed BTMs in obese subjects and most, except for TRACP-5b, in the control group. In the combined group, BAP was no longer significantly associated with leptin.

10.5.3 Childhood obesity and OC (III)

Subjects with a history of childhood obesity before the age of 7 years were still obese (BMI > 40 kg/m^2) as adolescents and during early adulthood (mean age 19 years), and 55% of the subjects presented with early signs of increased insulin resistance and hyperinsulinemia. In this group, the elevated fasting insulin (>12 mU/l), lower adiponectin (7734±3115ng/ml vs. 11625±6499ng/ml, p=0.004 for obese and controls, respectively) and 5-6-fold elevated leptin concentrations (p<0.001) together with more frequent smoking (> 1/3 being smokers), in addition to obesity and yet to be confirmed pre-diabetic status, predispose the subjects to an early risk of acquiring T2DM even with or without the speculated effect of OC.

In Study III, as concluded before, the obese subjects had lower baseline concentrations of BTMs, including OC and cOC, (other than BAP) compared with normal weight subjects (Table 3). As expected, acute glucose intake suppressed various BTMs as a sign of reduced bone turnover during OGTT but not the total OC behaved differently in controls vs. obese subjects (see 10.3.1). Additionally, the postprandial effect on total OC appeared unexpectedly later in obese subjects than in controls. Therefore, again, the results of Study III do not directly support the previously hypothesised presence of a short-term feedback loop between OC and energy metabolism in subjects with predisposition to or presence of a metabolic condition.

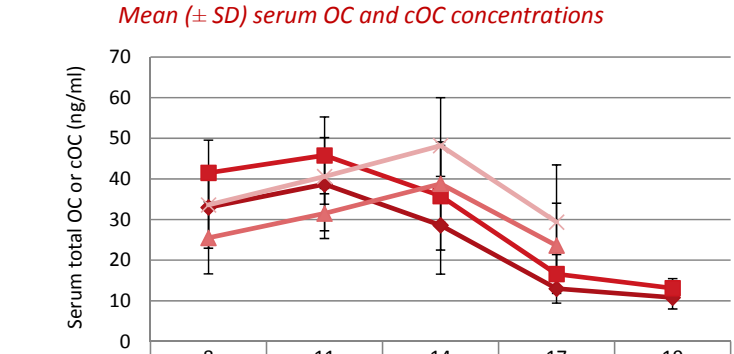
10.6 Normal paediatric reference ranges (IV)

10.6.1 Serum osteocalcin

The mean overall OC concentrations for girls and boys in Study IV are presented in Table 3. All 172 subjects in Study IV had serum samples drawn at fasting state and the mean (±SD) total OC and cOC values per age category for both sexes are presented in Figure 13. As expected, the peak serum total OC values per age category were observed at different time-points for the two sexes: 38.7± 11.47 ng/ml at 11 years for girls and 38.8± 9.43 ng/ml at 14 years for boys. The cOC concentrations followed consistently the same pattern (Figure 13). The largest variation within an age category (measured with the highest SD) was observed in age categories 14 and 17 for girls and boys, respectively. This observation is probably related to the mixed pubertal status in these categories vs. the earlier or later age categories.

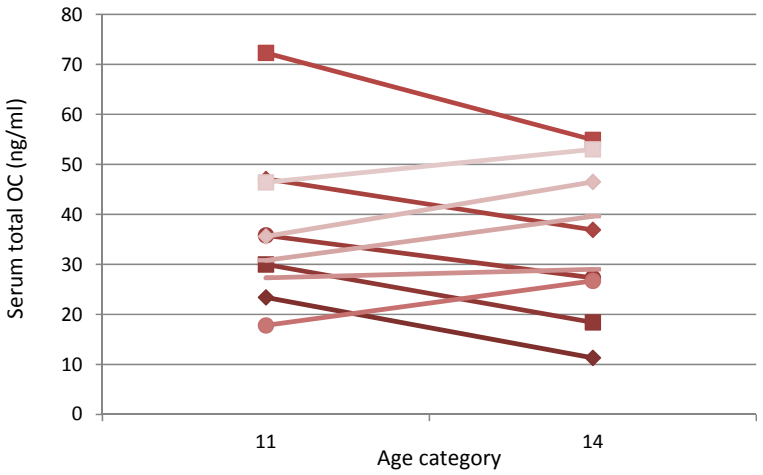
As expected, serum OC and cOC increased profoundly with puberty, with up 350-450% vs. the final late adolescence concentrations, and the values correlated with the pattern of pubertal growth, which started to decline earlier in girls (after 11 years) than in boys (after 14 years). The graphical presentation (Figure 14) of two age categories, 11 and 14, which coincide with the attainment of mid-puberty status, demonstrates how the timing between the age category-dependent elevation and decline in total serum OC varies between sexes, showing an opposite direction of dynamics on the level of the overall cohort, from lowest to the highest observed values, for girls vs. boys. The dynamics of the development of the total OC and cOC concentrations from childhood through adolescence are described in details in Section 10.2.

Figure 13: Mean±SD serum total OC and cOC concentrations per age category for both girls (darker red) and boys (pink tones)



	8	11	14	17	19
GIRLS OC	33	38.7	28.6	13	10.8
GIRLS cOC	41.5	45.8	35.8	16.6	13.1
BOYS OC	25.5	31.5	38.8	23.6	
BOYS cOC	33.6	40.6	48.2	29.4	

Figure 14: Dynamics of the opposite trends for minimum, 25th, 50th, 75th percentiles and maximum values of serum total OC for girls (dark red tones) and boys (pink tones) for age categories 11 to 14.



For the purpose of defining normative and clinically useful paediatric reference intervals the total serum, carboxylated and urinary OC values, Tukey's Hinges percentiles (25th, 50th and 75th) and graphical presentation of the combined minimum, maximum values for age categories, and separately for both sexes were prepared. The complete data are not shown here (refer to the submitted manuscript; see Figure 14 for minimum, 25th, 50th, 75th percentiles and maximum values for age categories 11 and 14 for total OC).

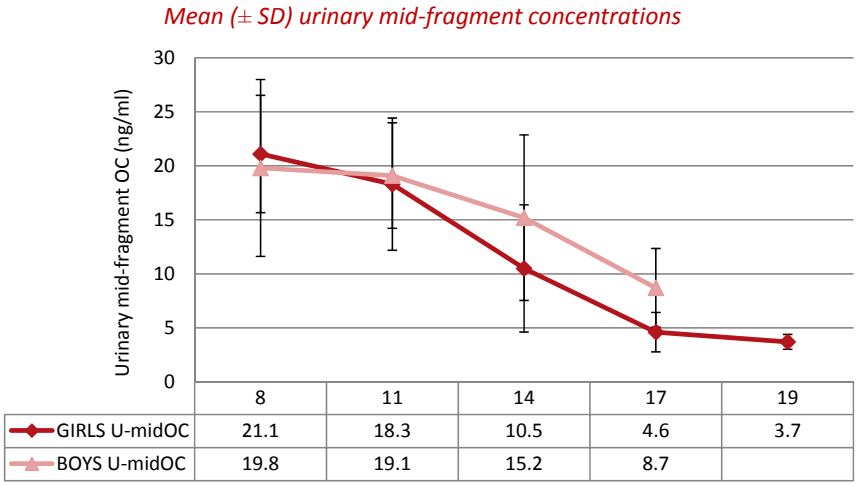
In a multiple regression analysis, the independent determinants of OC concentrations were age ($p<0.001$) and height ($p=0.003$) while cOC values were additionally influenced by weight ($p=0.037$) and PTH ($p=0.023$). Weight and Vitamin D concentrations demonstrated borderline significance for OC in the same analysis ($p=0.059$ and 0.076, respectively).

10.6.2 Urinary mid-fragment osteocalcin

Despite the presumed acceptance for collection of urinary samples (vs. serum samples), urinary samples for assessment of U-midOC were missing from 9 girls and 4 boys, i.e. the reference values were defined only in 159 children and adolescents. All the crude U-midOC values were corrected for urine creatinine before analysis. The mean (± SD) concentrations for each age category and for both sexes are presented in Figure 15.

Contrary to the serum OC and other BTMs, the creatinine-corrected U-midOC values were already elevated in pre-pubertal subjects, equally in girls and boys, in the lowest age category (8 years). Similarly, the U-midOC concentrations were comparable between both sexes in age categories 8 and 11 while the concentrations declined earlier in girls vs. boys, following the pattern of the serum OC/cOC values. In general, there was a significant correlation between the serum total or cOC and U-midOC concentrations in the overall pooled cohort for girls (Spearman correlation, all p-values <0.001), while for boys the pooled U-midOC concentrations lacked correlation between total serum OC and cOC concentrations (r=0.12 and r=0.234, for OC and cOC, respectively, p=ns). The correlation between the total serum, cOC and U-midOC progressively improved with increasing age especially in boys and reached significance in the oldest age (17) category (correlation for U-midOC: r=0.692, p=0.009 and r=0.610, P=0.027 for total OC and cOC, respectively).

Figure 15: Mean (± SD) urinary mid-fragment concentrations for girls (red) and boys (brown) per age category



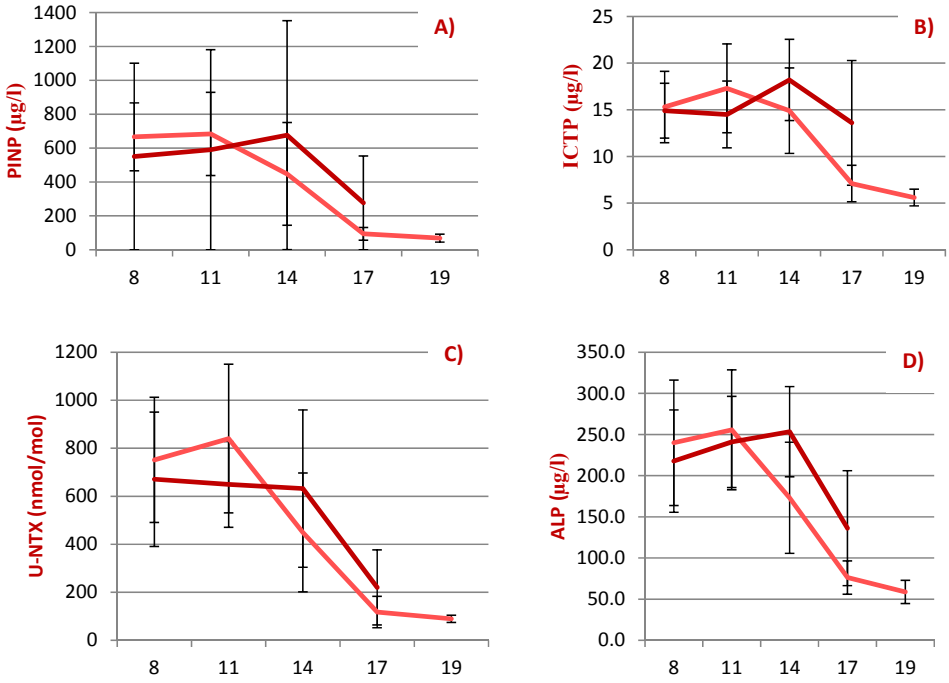
	8	11	14	17	19
GIRLS U-midOC	21.1	18.3	10.5	4.6	3.7
BOYS U-midOC	19.8	19.1	15.2	8.7	

U-midOC as a parameter was more robust vs. serum OC variants in a multiple regression analysis, and in the crude analysis based on evaluation of age categories, and only age (p=0.005) and puberty (p=0.030) were displayed as independent determinants of U-midOC.

10.6.3 Other BTMs

The mean ±SD reference BTM data for PINP, ICTP, urinary NTX and ALP are presented for both sexes per age category in Figure 16. The concentrations of all these reference BTMs increased with, or were already elevated prior to puberty, in both girls and boys. The BTM dynamics also correlated with pubertal growth with concentrations starting to decline earlier in girls (after 11 years) than in boys (after 14 years) following the pattern of OC and cOC. Supporting the DXA findings the reference BTM concentrations (PINP, ICTP, uNTx and ALP) for all the age categories confirmed that the subjects had a normal bone turnover at the time of evaluation, despite the generally low Vitamin D levels. Independent of the Vitamin D tertile, the reference BTMs were strongly associated with OC, cOC and U-midOC (p<0.001 for most BTMs, only ICTP not being associated with U-midOC in the highest Vitamin D tertile).

Figure 16: Paediatric mean ±SD reference BTM concentrations for PINP (A), ICTP (B), U-NTX (C) and ALP (D) in girls (pink) and boys (dark red)



11. Discussion

The overall purpose of this research was to explore whether, how and potentially why a bone-specific molecule, such as OC, is or is not involved in the suggested bi-directional regulation of energy metabolism. Additionally, we pursued to establish sex- and age-specific local Finnish BTM reference intervals, focusing on urinary, serum total and carboxylated OC levels, in healthy Finnish children and adolescents. As the proposed association between OC and glucose homeostasis has been explored mainly retrospectively, in *post hoc* analyses and in cohorts that included patients with multiple confounding factors, it was important to address the hypothesis in healthy young adults or those with metabolic syndrome or signs of it but no diabetes. Aspects related to the timing and magnitude of the changes in glucose but also OC homeostasis, whether these changes are biologically significant in normoglycaemic subjects, and how these changes associate with skeletal parameters, were still to be explored.

In addition, there has been inopportune use of causal language and lack of appropriately designed prospective studies to generate evidence regarding the role of OC in regulation of human glucose metabolism. Based on the present evidence, it is indeed debatable if any form of OC has potential as a therapeutic target for regulation of glucose metabolism in humans.

11.1 Translating science from mouse to humans

The appropriateness of the engineered mouse models for the study of OC as a therapeutic target in humans appears ambiguous due to various reasons. In normally fed mice, circulating OC is fully carboxylated, whereas in humans the proportion of circulating OC is lower, about 20%[146] compared with other species and only half of the circulating human OC is carboxylated[188]. The suggested, rather modest, changes in the concentrations of unOC in mouse models[159] indicate a very narrow therapeutic range of effect in animals, which has limited applicability in humans due to species-specific differences. There are also other critical species-specific differences in OC between mice and humans, such as only limited (60%) homology between the genes encoding OC in mice and humans[189]. In addition, the role of 1,25-Vitamin D varies in the regulation of the OC gene between these two species; the single copy of the OC gene in humans is upregulated whereas the three murine copies of the gene are downregulated by 1,25-Vitamin D[146,190]. In conclusion, these fundamental species-specific differences in the OC protein necessitate caution when translating the claims based on engineered mouse-models into humans.

11.2 Glucose, insulin and OC

11.2.1 Impact of acute glucose load on markers of bone turnover

We studied the suggested association between energy metabolism and OC and its bi-directional regulation by exploring the impact of acute glucose load, OGTT, on OC, cOC and other BTMs in healthy young adults and those with a history of childhood obesity. Our results indicate that an acute glucose load induces a significant and rapid decrease in serum OC, its carboxylated form and in markers of both bone resorption (βCTX-I, TRACP5b) and formation (PINP, BAP). The observed OGTT-induced decreases in these different bone-derived proteins are at least partially independent of each other, demonstrate different timing of maximum suppression and, at times, even inconsequent patterns between

the studied populations. Whether these acute changes are directly insulin-induced or mediated by OC or, as suggested, by unOC, remains unclear.

Different bone markers and their serum or urine levels reflect different steps of the constantly ongoing, coupled bone remodeling[191]. BTMs reflect the rate or speed pf enzymatic activity of the diverse bone cells, excess products from the formation or fragments released during the degradation of bone matrix components[192], [193], and thus circulating proteins or their fragments detectable in serum can be derived from various sources. Altering gene expression might influence the de-novo biosynthesis of the intact proteins and most BTMs demonstrate dependence on circadian rhythm, diet, age, concomitant diseases and medication and many more[21]. Thus, impact on any single factors on levels of BTMs should be interpreted with caution and concomitant assessment of multiple markers, preferably both resorption and bone formation BTMs provide with a more reassuring perspective. However, as observed in our studies, acute suppression of bone turnover and its markers after intake of nutrients is not a new phenomenon; diet-induced, non-insulin-mediated changes are usually greatest in the most commonly used BTM, serum βCTX-I levels[194],[195],[196] and of smaller magnitude[195],[197] or not statistically significant[194], in serum total OC. Both an OGTT and intravenous glucose tolerance test (IVGTT) have induced a decrease in CTX and total OC concentrations in otherwise healthy postmenopausal women while OGTT induced a significantly larger decrease in CTX than the IVGTT[196]. Whether the differences observed between OGTT and IVGTT were related to the dose of glucose or route of administration affecting the gastrointestinal incretin effect after oral dosing, cannot be determined. In our studies, OGTT-induced suppression in CTX was approximately 50-60%, depending on the population, and similar to the effect reported previously by others as a result of food intake[195],[198] and an oral glucose load[195].

Feeding has been reported to have very little effect on serum TRACP-5b when it was analysed at fed and fasting state[199]. Interestingly, in our young adults (Study I) an OGTT induced a significant reduction (median -44%) in serum TRACP5b levels while the adolescents with history of childhood obesity displayed a more typical, diminished response in TRACP-5b. Oral glucose load induced a smaller but highly significant reduction in PINP and a variable response in OC. Changes observed in PINP and OC were highly correlated to each other, but appeared to be independent of the suppression of bone resorption, as change in CTX-I was only moderately associated with the reduction in PINP and not associated with the changes in OC. No associations were observed between changes in TRACP5b and bone formation markers. Thus, these rapid changes cannot be explained by coupling between formation and resorption during the 120-minute time period.

Insulinotropic peptides, such as the already therapeutically utilised glucagon-like peptide -1 (GLP-1), GLP-2 and glucose-dependent insulinotropic peptide (GIP) have been studied as potential mediators of the postprandial reductions in BMTs. These incretins are released from the duodenal K cells in response to carbohydrate intake[200] and are known to be induced by feeding and, even more pronouncedly, by an OGTT as demonstrated in the classic incretin test by *Nauck et al*[201]. In animal studies, however, GIP has been observed to both inhibit bone resorption and stimulate bone formation[202]. Previous studies in healthy subjects have shown no direct effect of GLP-1 or GIP on serum total OC after ingestion of macronutrients or after exogenous parenteral administration of incretins[194],[203]. Only the bone resorption marker CTX was reduced as a response to a macronutrient meal or to exogenous GLP-2 infusion. These studies exclude both GIP and GLP-1 as key mediators for the immediate reduction in bone resorption after a meal[203]. Thus, the OGTT-induced suppression of serum OC and cOC observed in these studies is likely to be mediated via a different mechanism. On a general note, direct comparisons of the mechanisms and effects of an OGTT and ingestion of macronutrients on bone are debatable because regulation of glucose

metabolism under rapidly applied, non-physiological glucose load might be induced by different mechanisms than under more physiological intake of nutrients during a meal.

The observed inverse associations between the glucose levels and bone-formation marker BAP or the glucose and resorption marker CTX in our study indicate that postprandial hyperglycaemia or OGTT-induced changes in general suppress bone turnover, independent of the utilised BTM. Rather than exploring the regulatory role of a single bone-derived molecule, such as unOC, in glucose homeostasis, the association between OC and glucose may be indicative of a universal role of bone in glucose impairment and diabetes[175]. Similarly, earlier studies have proposed that hyperglycaemia may contribute to lower bone turnover[204] and favour differentiation of mesenchymal stem cells into adipocyte lineage instead of bone cells[205]. The unique response of OC to glucose during OGTT could have a logical explanation as OC is considered a secondary marker of late stages of bone turnover, produced by mature osteoblasts during the mineralisation process to stabilize the structure of the bone mineral, HAP[206]. Deletion of insulin receptor specifically in osteoblasts has been demonstrated to result in decreased secretion of OC in mice[207] and thus the delayed decline in OC noted in young but obese subjects could result from already suppressed baseline bone turnover, which in turn was further suppressed during OGTT.

11.2.2 Studying insulin signalling and role of OC

When evaluating the impact of glucose on post-load OC and cOC values in our main study population, these parameters correlated only weakly with postprandial glucose or insulin values indicating that OC does not seem to be the main mediator of acute glucose regulation in humans during OGTT. As discussed above, this is contradictory to the data from animal studies suggesting that insulin signalling in osteoblasts increases the secretion of OC, promotes glucose homeostasis via unOC and may thus prevent the development of insulin resistance, glucose intolerance and abnormal weight gain[132,154,157]. Several mechanisms based on murine data have been proposed, including stimulation of osteoblast differentiation and OC production, and increased release of unOC from the bone matrix due to increased bone resorption via alterations in RANK-RANKL-OPG pathway[154,155]. Yet, based on our findings and those from others (Table 1) it is increasingly unclear if a similar regulatory system is present in humans[146,147].

Our results have also raised a question about the best clinical method to evaluate the effect of OC on glucose homeostasis and especially physiological insulin release. As it is not biologically feasible or ethical to alter the levels of OC or to introduce a knock-out design in human subjects, the hypothesis of a bi-directional regulation of energy metabolism by bone can only be studied by altering the magnitude of the energy load instead. Alternatively, the hypothesis could also be explored by including subjects with potential predisposition to an altered OC metabolism or carboxylation status such as cohorts of prematurely born subjects with documented impairment in bone metabolism. However, it is not generally known whether clinically meaningful changes in markers of bone homeostasis or presumably rapid effect of insulin should be studied in a standardised 2-hour OGTT. On the other hand, more sophisticated approaches with continuous glucose monitoring e.g. in clamp studies in humans isolating the effect of insulin on bone turnover markers have also failed to confirm the effect of physiological changes in insulin on bone metabolism[197,208]. The same applies to clinical studies; as an example, in a cross-sectional study in an elderly population, the explored effect of OC was significantly correlated with insulin resistance but no association with insulin secretion was observed[140]. Our results do not, however, exclude the possibility that insulin-induced OC may regulate glucose and bone metabolism over a longer or shorter period of time than the intervals used in these studies. Our data simply indicate that, in humans, OC and

cOC levels rapidly and significantly decrease following the glucose load, but the magnitude or the timing of the change is not associated with increases in insulin levels.

In summary, our results confirm that, in humans, acute postprandial changes in glucose homeostasis are still primarily regulated by the previously well-established physiological, non-skeletal and endocrine regulation pathways beyond those suggested for inclusion of OC[209,210,211]. The difference between the results observed in engineered knockout mice and in humans regarding the proposed role of OC in regulation of glucose homeostasis can potentially be attributable to the species-specific differences in OC[189,190]. The regulatory role of bone metabolism and, in particular, OC on glucose and insulin metabolism may also be different in acute and chronic metabolic challenge situations in humans.

11.2.3 VLBW status, glucose and OC

As only a limited number of studies have evaluated bone metabolism in VLBW subjects, the selection of our cohort with predisposition to a presumed impairment in OC metabolism provided additional opportunities for mapping the bone health of VLBW subjects beyond the general research question regarding the endocrine role of OC. Some studies have reported increased OC in male VLBW subjects[212] while, when exploring BTMs, another study identified no significant differences in formation or resorption markers[213]. In our study, OC and cOC were higher in subjects born with VLBW, and elevated OC levels could partially explain the observed decreased insulin sensitivity in VLBW subjects. Our finding, again, is somewhat contradictory to previous, mostly preclinical findings in mice on the role of OC in energy metabolism[132,154,157].

The baseline insulin and OC values were higher in female VLBW subjects than in females born at term but as previously reported, there is no general, significant interaction between genders in this VLBW cohort[122]. Our data indicate that the gender-specific differences do not significantly impact the suggested role of OC in acute postprandial glucose regulation. Subjects with VLBW are smaller, shorter and have lower BMI than their peers, and thus, the glucose load and total dose of glucose is proportionally higher for them. Also subjects born at term (but small for their GA) have been shown, in observational studies, to present with signs of impaired glucose regulation in adulthood[214,215]. VLBW subjects have reduced BMD as young adults. This might be due to their smaller size even if the measurements are corrected for bone size by estimation of volumetric bone density[123,213]. However, additional adjustment for anthropometric or other baseline clinical characteristics such as BMI, lean body mass and fat-% did not significantly alter the results, and thus, changes were mainly not attributable to body size or body composition. This is in agreement with results indicating that early signs of decreased insulin sensitivity, body size and composition did not explain the difference in regulation of insulin[213].

11.3 Effect of metabolic impairment on bone

During hyperglycaemia

Patients with T1DM have inadequate accrual of peak bone mass and impaired bone formation[216], while T2DM patients typically have aberrant, either increased or decreased BMD[217]. When studying the basic physiological effect of any bone-derived components on energy metabolism or glucose on BTMs, it is crucial to exclude the potential metabolic confounders by selecting an apparently healthy normoglycaemic population, such as our young adults with or without a history of VLBW. Additional

confounding factors, such as T2DM -related comorbidities and complications, might influence BTMs, and some of the administered anti-hyperglycaemic medications such as thiazolidinediones shift the development of the mesenchymal stem cells and increase fracture risk in subjects with glucose impairment or T2DM[218]. In our cohort of subjects burdened by childhood obesity, the effect of the past metabolic disturbance was displayed as reduced BTM levels at the time of evaluation. Generally, lower BTMs have been observed in patients with T2DM vs. T1DM[219,220]. Although it is unknown why bone turnover markers are lower in patients with T2DM it is speculated to be related to the insulin resistance characteristic of obesity and T2DM[219].

The sustained elevated plasma glucose levels may affect OC and other BTMs directly or through mechanisms of insulin, incretins or other gastrointestinal hormones. This secondary effect to glucose and elimination of the effect of the OGTT has been demonstrated with octreotide, an inhibitor of insulin effect, incretins and gastrointestinal hormones[195]. Alternatively, insulin-mediated glucose uptake in osteoblasts and osteoclasts might also directly decrease the BTMs. Ominously elevated glucose levels, which can only be tested in *in vitro* setting, can be used to feature the effect of glucose on bone turnover. Multiple mechanisms exert the deleterious effect of high glucose on markers of bone turnover[221,222,223]. Sustained hyperglycaemia may affect the bone by suppressing bone resorption, increasing production of un-mineralised bone matrix or decreasing the degree of mineralisation[224,225]. Such processes can be assessed by determining the rate of pit formation in osteoclasts, production of Type I collagen in osteoblasts or by measuring ALP. Hyperglycaemia and advanced glycation end products (AGEs) can also promote the apoptosis of osteoblastic cells[226,227]. It must, however, be kept in mind that these dramatically increased glucose levels used in experimental setting are non-physiological for most of the diabetes patients under treatment (over 30 mmol/l or beyond), and studies using modestly elevated, but unfortunately not so uncommon, glucose concentrations of 10–15 mmol/l of glucose, are indicated.

In T2DM patient populations, pronounced glycaemic variability, due to lack of sufficient glycaemic control or treatment-emergent fluctuation of daily glucose values is commonly observed[228]. Relevant glycaemic variability might also be reflected in the pattern of regulation of bone turnover in subjects with diabetes: inducing a state of cyclically varying bone turnover, where bone turnover is decreased at high glucose levels and normal at normal glucose levels. In this context, the assessment of HbA1c value as a sign of glycaemic impairment may not be as relevant as the instant glucose value, and assessment of variability at the time of blood sampling, such as during OGTT, displays the dynamics of the BTMs[195,196]. The HbA1c only reflects the average blood glucose concentration during the last 4-12 weeks and even if the value is weighted towards the last 2-4 weeks, reliable signs of change can be demonstrated at earliest from 20 days onwards (when e.g. efficacious glucose lowering is being implemented)[229] while the plasma glucose values may at times during these 3 months be variably high or low, especially at fasting state. Thus, an association between a BTM and HbA1c values at a randomly chosen time-point might be of limited relevance.

In subjects with T2DM, adiposity, through inflammatory cytokines, adipokines and free fatty acids has a long-term detrimental effect on bone but may also give rise to a chronic low-grade inflammation that may additionally suppress bone turnover and lower BTMs. A significant, negative correlation between BAP and tumour-necrosis factor alpha (TNF-α) has been shown, whereas correlations between OC and inflammation markers all were insignificantly negative[230]. In our cohorts, the relationship between OC and adiponectin was also unclear. Another study yielded contradictive results; OC was negatively correlated with inflammatory markers such as interleukin-6 and high-sensitivity CRP (hs-CRP)[231]. As the role of chronic inflammation and its effect on bone in subjects with T2DM is still under investigation the available evidence is relatively scant.

Changing glycaemic control and pre-diabetes

On the other hand, improved glycaemic control and return closer to normoglycaemic range might stimulate osteoblastic differentiation and enhance bone formation. In patients with T2DM, total serum OC and its ratio against BAP (OC/BAP) correlated negatively with HbA1c values and positively with IGF-1 in Japanese male subjects[120]. However, there are several studies which have shown low, normal and high IGF-1 levels in obese, normoglycaemic subjects with different degrees of insulin resistance, and thus the correlation with IGF-1 deficiency might not be the only relevant mediator between glucose and bone metabolism[232],[233],[234],[235]. Studies with obese adolescents presenting with metabolic syndrome have shown a negative correlation between BMD and insulin levels or insulin resistance as assessed by HOMA-IR in e.g. Brazilian subjects[236]. An inverse association between serum leptin concentration and BMD, adjusted for body mass, was detected in the same study indicating that leptin, insulin and HOMA-IR could be linked to bone metabolism in these subjects. However, this finding could not be repeated in another trial suggesting that serum leptin would not be a direct determinant of BMD at least in Chinese population[237], and thus the role of leptin as a determinant of BMD-related parameters is not very conclusive, or the variability is dependent on ethnic factors.

The dynamic role of OC as a potential mediator in these populations with mild hyperglycaemia has not been properly assessed. In a smaller Japanese study cohort (n=50 patients with poorly controlled T2DM) improvement of glycaemic control was associated with a decrease in BAP and an increase in total OC[120]. Changes in HbA1c were inversely associated with changes in OC, including the proportion of unOC in relation to total OC (unOC/OC), leaving space for speculation over the potential role of decreased unOC as a marker of improved, previously impaired bone formation.

After introduction of a lifestyle intervention or dietary modification

Peripheral glucose sensitivity and insulin secretion have been demonstrated to improve under strenuous or interval exercise[238],[239]. Circulating insulin levels decline during physical exercise while insulin delivery is increased or at least maintained at the same level with increased skeletal muscle blood flow[239]. Also reduced burden of hyperlipidaemia favourably influences insulin signalling and glucose sensitivity[240]. The proposed correlation between TGs and OC or change in either parameter, has been reported with variable results; a negative association was induced with diet or diet plus resistance training intervention during which circulating levels of OC were associated with insulin sensitivity, mainly in lean subjects, and with insulin secretion[170]. Previous attempts to induce changes in levels of OC with caloric restriction have failed[171] but potentially bone loading could lead to increased synthesis of OC; the question remains whether the proportions of carboxylated and uncarboxylated OC remain the same under loading. In another study, obese, but otherwise healthy, post-menopausal women undergoing a supervised 20-week weight loss program were studied to determine the associations between changes in total OC and degrees of carboxylation of OC and changes in body weight and percent body fat (BF%)[241]. All participants received supplemental Vitamin K, D and calcium over the 20-week intervention period. After loss of an average 10.9 ± 3.9 kg and 4% body fat, the serum concentrations of total OC, unOC, percentage of unOC and PINP did not significantly change, nor were these measures associated with changes in weight or BF% (all p=ns). These data do not support an association between OC and weight or BF% loss in post-menopausal women supplemented with nutrients implicated in bone health[241] and adds to the conflicting pool of evidence showing opposite results vs. other studies exploring the proposed hypothesis.

Vitamin K has been established to be a key player in carboxylation of OC[131],[136]. No association between unOC and insulin resistance, as measured by HOMA-IR, has been demonstrated in studies with Vitamin K supplementation[140],[242].

Bone turnover cycle

In our ELLU study population, reduced OC but similar BAP baseline concentrations were reported for those subjects with a history of childhood obesity vs. their controls. Comparable findings were reported in a study addressing levels of BTM in T2DM compared to controls. Similarly, total OC was decreased and hydroxyproline, a component of collagen less frequently used as a resorption marker, was increased among subjects with T2DM compared to those without diabetes, whereas BAP concentrations did not differ between groups[243]. Even if our obese subjects were still considered normoglycaemic, they demonstrated early signs of insulin resistance, the most well-established determinant and driver of pathophysiology of T2DM in an obese population. Thus, based on the BTM pattern, the hyperinsulinemia and temporary hyperglycaemia may have affected different stages of the bone turnover cycle. These differences in BTMs may explain the general paradox of lower bone strength and increased BMD in T2DM, as markers of bone resorption and formation seem to be lower, whereas the mineralisation, which is reflected by BAP, is within normal limits or not different from the controls[244],[245]. The lower bone turnover may not be coupled to a similar lowering in mineralisation, making the bone matrix hypermineralised, and leading to a more frail and almost osteopetrotic bone[246]. Thus, additional adjustment of BTM reference range data for metabolic factors and adiposity (BMI, body composition) but also exclusion of subjects with diabetes or glucose impairment provides the foundation for a more appropriate interpretation of the BTM results in apparently healthy subjects.

Future fracture risk and BTMs in subjects with glycaemic impairment

In our obese subjects (ELLU cohort), the observed low bone turnover in relation to higher skeletal area in young subjects is an important finding as it may have multiple consequences, including an increased risk of future fractures. Increasing adiposity has been suggested as a risk factor for fracture in children, with[247],[248] or without fractures[249], and low BMD, consistent with an increased fracture risk, has been observed in patients with T1DM[250]. BMD is considered as a golden standard for evaluating the risk of fractures in osteoporotic populations, while assessment of an amount of mineralised bone might be sub-optimal for fracture risk assessment in subjects with glucose intolerance or diabetes. On the other hand, cross-sectional studies examining fracture risk and BTMs have been conducted with different outcomes; serum OC/BAP ratio has been shown to be inversely associated with the presence of vertebral fractures (OR=0.695, $p<0.05$) even after an additional adjustment for lumbar or FN BMD[251]. However, lack of glycaemic control and lower IGF-1 levels may cause osteoblast dysfunction independently of BMD and thus indirectly contribute to reduced bone quality and fractures. Reduced or low bone turnover, marked by decreased BTM levels such as IGF-1 or OC, or increased sclerostin levels, or combined low PINP and elevated CTX concentrations, are associated with vertebral fractures in T2DM[252],[253],[254],[255],[256]. Moreover, elevated u-NTX has been shown to be increased in insulin-treated women with T2DM and post-menopausal osteoporosis and a vertebral fracture[257]. Nevertheless, there is also increasingly opposite evidence highlighting the uncertainties regarding assessment of OC as a reliable marker in subjects with T1DM and long-term or insulin-treated T2DM with complications. Lack of association between OC and other BTMs and vertebral fractures have been published by many[120],[255],[257],[258],[259]. In

general, in subjects with insulin-treated diabetes any results should be interpreted with caution as especially IGF-1 levels may be altered due to insulin treatment and severity of diabetes.

Risk of diabetes during or after use of anti-resorptive therapy

When reconsidering the original theory by *Karsenty et al* [132] regarding the role of bone in regulation of energy metabolism, one of the most striking consequences of the theory is the presumed increased risk of metabolic diseases due to use of anti-resorptive therapy. If initiation of bone resorption was crucial for conversion of OC into its uncarboxylated form, then therapy-induced suppression of bone turnover, e.g. with bisphosphonates, should lead to an increased risk of hyperglycaemia and secondary diabetes. However, none of the independent, pivotal osteoporosis trials, with detailed assessment of both skeletal and glycaemic parameters, have reported an increased risk of T2DM or any impediments in glucose tolerance after intervention with anti-resorptive therapies[260]. The same also applies to smaller studies in which OC and unOC and glycaemic parameters were assessed in detail in subjects receiving other drugs known to alter bone turnover, such as PTH or hormone replacement therapies[261,262,263,264,265]. Interestingly, data from the national Danish registry reported a reduced incidence of risk of diabetes in those on anti-resorptive therapy[266], albeit the registry has no information about the OC levels of the targeted individuals. In conclusion, at least in humans, altering the rate of bone resorption either does not lead to a sufficient increase in circulating forms of unOC and/or the elevated unOC does not significantly influence glucose metabolism.

11.4 The role of OC and other BTMs in healthy, younger subjects

Age, height and weight, and even PTH and puberty, are independent determinants of serum and urinary OC levels during childhood and adolescence. Other BTMs are expectedly influenced by age, sex and pubertal stage, as the peak concentrations of all BTMs, including OCs, followed the well-established pattern also shown by others[88,89,90]. Other variables, including key biochemical markers reflecting overall health status, seem to have no or limited impact on these BTMs. Similarly, and rather unexpectedly, DXA parameters show limited or no impact on the assessed BTMs. While in adults the BTMs mostly predict and reflect current BMD values, the ongoing rapid modeling process and thus elevated BTMs in children and adolescents potentially only predict their future, adult BMD status rather than correlate with a relatively random BMD value at a given time-point during a paediatric growth spurt. Consequently, BTMs indicate bone turnover at the time of evaluation while BMD is a marker of long-term effect reflecting the events of the preceding months or even years.

Limited paediatric reference data available

Our cohort contained a reasonably homogenous and representative group of young subjects with normal bone health, as indicated by reference BTMs[89], and scattered over different stages of pubertal growth spurt. Most of the available paediatric BTM data and guidance for interpretation of the results in children and adolescents is only applicable to post hoc analysis of birth cohorts, different bone-derived diseases, osteoporosis or conditions inducing a secondary effect of glucocorticoid therapy on their bone health[249,267,268,269,270,271]. In those populations, assessment of calcium, PTH and phosphate levels in relation to the BTMs has been demonstrated to be crucial for correct interpretation of impaired bone markers and metabolism in children and adolescents. In our cohort, only PTH, and not Vitamin D, along

with expected age, height and weight were assessed to be independent determinants of cOC. Thus, the role of biochemical markers, including calcium or phosphate levels in predicting OC concentrations seems limited in healthy subjects and only becomes more substantial if BTMs are used to assess skeletal health when preventing any secondary effects such as osteoporosis or growth hormone deficiency in paediatric cohorts. Our cohort additionally displays important and expected characteristics typical for a Finnish population, including low Vitamin D levels in midwinter samples, providing a platform and reference intervals for clinical interpretation of BTM results in corresponding subjects, with the seasonal midwinter nadirs. Statistically significant seasonal impact of variable Vitamin D concentrations on BTMs has been reported[267] but the clinical relevance of such small effect size is questionable when assessing the BTM concentrations in healthy children and adolescence.

Methodology- serum or urine sampling?

When assessing any biological markers in healthy individuals, but also, in children and adolescence with chronic conditions requiring long-term monitoring, the preferred sampling method is non-invasive and pragmatic, such as urinary sampling as an alternative to serum sampling. Paradoxically, in our paediatric healthy cohort the urinary samples were missing more often than any of the serum samples (100% adherence to sampling); involuntary production of urine at any given time indeed seems challenging in all age categories from infants to the elderly.

The mid-fragment OC, detectable in urine, reflects a different dimension of bone turnover, including resorption[272] while it has also been demonstrated to be a marker of growth in a special population of prematurely born infants during their growth spurt in the postnatal period[152]. Our study results indicate that even if the urinary OC concentrations were comparable between sexes in younger age categories there was a limited correlation between serum OC and urinary OC, especially in younger boys, and these values were mostly driven by age categories, with progressively improving correlation with increasing age. This might not be a unique finding as comparisons in post-menopausal populations between other BTMs in both urine and serum have revealed that urinary co-efficients of variation may be twice as high as markers detectable in serum[273]. A preferred and holistic assessment of bone turnover in this population would be a combined assessment of several urinary bone turnover markers for reduction of the effect of variation within an individual sampling time and age category. Additionally, urinary markers depend on the amount of marker secreted during bone formation and released from bone during bone resorption. Therefore, they are not directly translatable into amounts of bone gained during a growth spurt while a simultaneous assessment of bone markers displaying both the rates of bone resorption and bone formation will provide a more reliable, composite result of the overall bone turnover.

11.5 Validation- limitations and strengths of this research

The key strength of our chosen cohorts was that all the adult subjects were meticulously studied during the time when they were close to, or at the age of, their peak bone mass. The selection of these unique cohorts, especially the pooled population from Study I-II, provided an optimal platform to explore the alleged bi-directional regulation of energy metabolism without significant confounding factors. The methods also included a comprehensive analysis of bone metabolism by measuring multiple BTMs, targeting resorption and bone formation or its rate, and different forms of OC, cOC and U-mid-fragment OC, skeletal parameters by DXA, at various time-points during OGTT in obese young subjects or those born with VLBW vs. their carefully selected controls. Inclusion of these healthy populations without

substantial comorbidities and thus with special potential to confirm or reject the hypothesis regarding the presumed universal causality between bone turnover and energy metabolism, stood out as a novel and innovative study design.

Based on the preclinical data, the changes in the metabolic pattern, the effect size and its timing in our human subjects became increasingly unclear. This was partially due to the chosen approach, evaluation of changes in energy load on OC (vs. OC knock-out in animal studies) but also, as a standardised OGTT did not allow us to study the continuous direct effects of glucose or insulin on bone metabolism. However, it is important to remember that also attempts to confirm the role of OC in glucose metabolism failed when clamp techniques were implemented[208]. We did not have access to direct measures of unOC but assessed total OC and cOC and estimated the biological activity of OC and indirectly the effect of unOC, based on these values. In our assay the slopes of the standard calibration curves for OC and cOC did not run in parallel and thus the estimated method, deduction of cOC from total OC concentration, for unOC may not be sensitive enough to detect small changes in the carboxylation status of OC. Further, we did not have data on vitamin K intake or vitamin K status, which is known to influence carboxylation of osteocalcin[146]. On the other hand, it is unlikely that any of our subjects had severe vitamin K deficiency since they were not on parenteral feeding or any other dietary restrictions.

Feeding with an acute glucose dose gives rise to various carbohydrate-induced incretin hormones such as GIP and GLP-2, which might have partially confounded our findings for cohorts with OGTT. GLP-2 has been identified to directly inhibit bone resorption[194] and our results from all cohorts, without analysis of the incretin hormones, are aligned as the CTX values in particular were markedly suppressed during OGTT. A further limitation is that we measured only total adiponectin and not the isoforms, which associate more strongly with insulin resistance and metabolic syndrome. On the other hand, a standardized OGTT differs from a more physiological intake of nutrients, but otherwise corresponds to real life situation with an acute postprandial load, and thus represents the glycaemic variant of an exercise ECG: a stress-test of the metabolic response in an otherwise healthy body.

The main weakness of our cohort with subjects with severe childhood obesity but also, the paediatric normal reference range study, was the limited overall number of subjects: for normal ranges a total of 172 children and adolescents are less than recommended by international guidelines for validated assessment of reference intervals[89]. Specifically the low number of boys and in general, the partially uneven distribution of pubertal stages over the two sexes might explain the low correlation between the serum and urinary OC values. On the other hand, we have established reference ranges in an otherwise representative cohort of healthy girls and boys, through different stages of puberty and an array of BTMs reflecting key dimensions of bone formation from childhood towards adulthood and peak bone mass. When defining reference ranges for local clinical implementation it is crucial for the cohort to display and mirror important and expected characteristics of a typical, in our case Finnish, population, including low Vitamin D levels collected in midwinter samples, providing therefore a platform and reference intervals for clinical interpretation of BTM results in corresponding subjects.

All total OC and cOC samples were analysed with the same method, mostly by the same individuals holding the micropipettes. The analysis results can therefore be considered suitable to be explored from the perspective of a more generalised and composite analysis – the development of dynamics of OC concentrations over time spectrum and age categories from childhood to younger adult ages in apparently healthy individuals.

11.6 Conclusions and future directions

This research explored the concept of an assumed link between glucose and bone metabolism and whether it could be demonstrated in a population, which displayed weak signs of early insulin resistance and impaired glucose tolerance in early adulthood, in an apparently healthy VLBW cohort, or in obese young adults without diabetes vs. their controls. The purpose was also to evaluate if changes in glucose load were reflected in serum OC concentrations and its degree of carboxylation. Furthermore, normal paediatric reference ranges and their independent determinants for serum total, carboxylated and urinary mid-fragment OC were to be established.

Based on the observations the following conclusions can be presented:

1. A significant decrease in serum concentrations of both total OC and its carboxylated form, cOC, after oral intake of glucose resulted in rapid suppression of other BTM, and bone resorption and formation markers in all young adults.

2. Young, obese subjects with early signs of increased insulin resistance have lower concentrations of BTM, other than BAP, compared with normal weight subjects at baseline. The postprandial total OC was suppressed but the OGTT-induced effect appeared later and with reduced magnitude in obese subjects versus their controls.

3. Energy metabolism evidently influences parameters of bone turnover but the direct role of insulin, as the mediator of these changes, needs further investigations: postprandial insulin did not associate with OC or cOC either before or after OGTT.

4. Age, height and weight, and PTH and puberty, are independent determinants of serum and urinary OC levels during childhood and adolescence. In addition, all reference BTM followed the expected, puberty- and growth spurt-related patterns in our representative Finnish cohort of healthy children and adolescents.

The most pragmatic and clinically applicable results of this thesis are unquestionably those related to the newly proposed BTM reference ranges in a representative Finnish cohort of healthy children and adolescents. These suggested reference ranges, applicable for growing children and adolescents, 7 to 19 year-old boys and girls, and with only a limited number of independent determinants for the urinary, serum total and carboxylated OC levels, provide with a tangible tool for clinicians assessing the bone turnover in both healthy youngsters but also in those with impediments of bone health, with or without treatment.

Our other results indicate that the accumulating evidence, including our involvement in the pool of systematically and objectively evaluated data in healthy humans, has weakened the hypothesis suggesting that OC is a key factor responsible for regulation of metabolic control or energy metabolism. Whether this is mostly due to species-specific differences, which preclude the direct translation of the earlier findings from the animal models to humans, is not yet known. Alternatively, the original theory needs refining as also our research has contributed to the increasing wealth of less confounded evidence questioning the validity of the original hypothesis linking OC as a key player in energy metabolism.

For clinical applicability, studies targeting the effect of therapeutically manipulated bone cells on glucose homeostasis (e.g. with both anti-resorptive or anabolic agents used in management of osteoporosis) or those changing the degree of carboxylation in a more physiological setting, as with the use of warfarin

or low vitamin K–containing diet, are still missing. The alleged role of OC in impaired glucose tolerance is additionally difficult to be studied in rodent models displaying hyperglycaemia and its consequences as the pathophysiological pathways leading to the complications of diabetes are different in humans vs. those observed in genetically engineered mice. It is also not feasible or ethical to mimic the knock-out design (vs. animal, mostly mice models) in humans and thus replication of the animal studies in human subjects cannot be completed. Therefore, the animal-derived data can only be hypothesis–generating while the exploration of the putative fundamental role of OC in energy metabolism must continue in human populations, which are preferably less burdened by confounding factors.

Total OC is and remains a reliable biomarker of bone turnover in subjects of any age. Bone is in a constant state of turnover, and the entire skeleton is regenerated every 10 years. Therefore, it has been essentially impossible to distinguish the role of OC as an established marker of bone turnover from its potential role as a mediator of glucose metabolism through the use of secondary analysis of completed osteoporosis populations. Interestingly, the observed inverse association between the crudely assessed bone turnover and insulin resistance in diverse patient populations were immediately interpreted as direct evidence that unOC is indeed a hormone involved in human glucose metabolism. Independently, there has been unprecedented, inappropriate and surprisingly non-scientific use of causal language in citing results such that the different forms of OC have been used interchangeably in the literature even though only the unOC was found to have an effect in the original mouse model.

As the scientific community is increasingly concluding, in line with our own findings, prospective clinical trials designed specifically to test the suggested bi-directional hypothesis are unquestionably required. Based on the evidence today, it is increasingly uncertain that OC or any biological form of it, such as unOC, has potential as a therapeutic target for regulation of glucose metabolism in humans.

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Päivi Maria Paldánius

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